REMARKS/ARGUMENTS

Amendments:

Claims 1-13 and 33 are pending. Claim 1 was amended to incorporate the recitations of claims 3, 4, 5, and 8. Support for the amendment to claim 1 is in original claims 1, 3, 4, 5 and 8.

Claims 3-5, 7-8 and 11-12 are canceled.

Claim 6 has been amended to depend on claim 2 - to correct a typographical error.

Claim 9 has been amended to depend on claim 1 instead of canceled claim 8.

Claim 10 has been amended to recite that the cooked composition is prepared according to the method of claim 1. Support for this amendment is in original claims 1 and 10.

Claim 33 has been amended to more clearly define the invention. Support for the amendment is in original claims 1, 3, 4, 5, 8 and 33. Further, claim 33 has been amended to correct a typographical error in numbering.

No new matter is added and the entry of the amendments is respectfully requested.

Claim Objections

The claims were objected to because two claims were mistakenly listed as claim 22. Applicants have canceled claims 12-32 to remove this inconsistency. Claim 33, erroneously listed as claim 32, has been amended to correct the typographical error. Since the pending claims, as amended, no longer contain duplicate numbers, withdrawal of this objection is respectfully requested.

Specification Objections:

The Examiner has objected to the specification as allegedly containing improper incorporations by reference (Office Action, page 2). The Examiner states that the instant application uses "omnibus" language and fails to teach with particularity where specific

information is to be found in each reference. *Id.* In support of this objection, the Examiner cited *Advanced Display Systems, Inc. v. Kent State Univ.*, 54 U.S.P.Q.2d 1673 (Fed. Cir. 2000) and also highlighted sections of *Advanced Display Systems, Inc.* that cited *In re Seversky*, 474 F.2d 671 (C.C.P.A. 1973) and *In re Lund*, 376 F.2d 982 (C.C.P.A. 1967). Applicants respectfully traverse this objection.

First, the *Seversky* and *Lund* cases are not applicable to the instant application. In the *Seversky* case, the Court of Customs and Patent Appeals held that the simple statement that a daughter application was a "continuation-in-part" of a parent application was insufficient to incorporate the parent application by reference. *Seversky*, 474 F.2d at 674. In the *Lund* case, the Court of Customs and Patent Appeals held that a single sentence in a pending application indicating that it was a "continuation-in-part" of an abandoned application was insufficient to incorporate the abandoned application by reference. *Lund*, 376 F.2d at 989. By contrast, the instant application contains clear language that the cited publications are "incorporated by reference" in the application. *See, inter alia*, page 10, lines 1-2 and page 28 last paragraph of the specification. Thus, *Seversky* and *Lund* are irrelevant to the instant case.

Second, Applicants have specifically described where the information is found in the incorporated references. According to *Advanced Display Systems*, the incorporated material must be described with sufficient particularity to "one reasonably skilled in the art." *Advanced Display Systems*, 54 U.S.P.Q.2d at 1680. The instant specification discloses the first author, journal, volume, year, and page numbers for the incorporated publications. As an example, the paragraph spanning pages 4, lines 19-24 of the specification states:

> FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology [see e.g., Anastasi et al., Blood 77:2456-2462 (1991); Anastasi et al., Blood 79:1796-1801 (1992); Anastasi et al., Blood 81:1580-1585 (1993); van Lom et al., Blood 82:884-888 (1992); Wolman et al., Diagnostic Molecular Pathology 1(3): 192-199 (1992); Zitzelberger, Journal of Pathology 172:325-335 (1994)]. However, several of these studies address hematological disorders where genetic changes

> are assessed in freshly fixed smears from bone marrow aspirates or peripheral blood specimens. United States Patent No. 6,573,043 describes combining morphological staining and/or immunohistochemistry (IHC) with fluorescence in situ hybridization (FISH) within the same section of a tissue sample.

Thus, the instant application discloses the first author, journal, volume, year, and page numbers for the Anastasi publications (6 pages each) and the van Lom (5 pages), Wolman (8 pages), and Zitzelberger publications (11 pages). It is respectfully asserted that one of skill in the art would know where to find these publications, and would be able to read the very small number of pages contained therein. Rather than "omnibus" language, the instant application uses specific citations that are accessible to skilled persons in the art.

Third, Applicants point to the level of particularity that is required by the courts. In *National Latex Products*, the Sixth Circuit held that a brief statement was sufficient for incorporation by reference: "A suitable apparatus for continuous internal casting is shown in the application of Henry Martin and Paul Rekettye, Serial No. 179,726 filed August 16, 1950..." *National Latex Products*, 274 F.2d at 230; U.S. Pat. No. 2,629,134, col. 4. In *In re Fried*, The Court of Customs and Patent Appeals held that a short statement was sufficient for incorporation by reference: "The...steroid reactants can be prepared as disclosed in the applications of Josef Fried, Serial Nos. 489,769 and 515,917, filed February 21, 1955, and June 24, 1955, respectively." *In re Fried*, 329 F.2d 323, 325 (C.C.P.A. 1964). Compared to these statements of incorporation, Applicants have certainly provided sufficient particularity.

For all of these reasons, Applicants respectfully assert that the specification includes proper incorporations by reference. Withdrawal of this objection is respectfully requested.

§ 112 First Paragraph - written description

Claims 1-13 and 33 have been rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement (Office Action, page 5). The Examiner states that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one of skill in the art that the Inventors

had possession of the claimed invention and raised a number of issues to support the rejection. *Id.* Each one of these issues is addressed below.

First, the Examiner states that the claimed method encompasses the preparation of fixed material from cells or tissue derived from any plant, as well as from any tissue found in any mammal, and therefore lacks sufficient written description (Office Action, page 8). For this rejection, the Examiner relies on the court's decision in *University of California v. Eli Lilly and Co.* 43 USPQ2d 1405 (Fed. Cir. 1997) (See, Office Action, sentence spanning pages 7 and 8). Applicants respectfully traverse as follows.

University of California is clearly distinguished from the instant case. In *University of California*, the claims at issue were directed to recombinant plasmid with an insert that encodes insulin. *University of California*, 43 U.S.P.Q.2d at 1401. The Court held that the claims required human insulin-encoding cDNA and such cDNA was not disclosed in the specification. *University of California*, 43 U.S.P.Q.2d at 1404-05. Because of this, the Court affirmed a lower court decision that the claims are invalid. Id. at 1405.

In contrast to the University of California case, Applicants' claimed invention is directed to methods of preparing tissues for *in situ* hybridization after the tissues samples have been prepared (See, e.g., claim 2 of the instant Application). In some instances, the prepared tissue is already fix treated (See, e.g., claim 1 of the instant Application). Therefore, the focus on the written description for such methods is misplaced. Applicants traverse this rejection because the claims are not directed to methods of preparing tissue samples or fixing tissue. See, e.g., independent claims 1, 10 and 32. Methods of preparing tissues from various cell types, including methods for preparing fix treated tissue, are not claimed by the pending claims. Since preparation of tissues and fixed treated tissue is not claimed, it is immaterial whether the instant specification provide adequate description of how to prepare tissues from various cell types.

Furthermore, the preparation of fixed treated tissue is well known at the time the instant application was filed (i.e., November 20, 2002). For example, methods for fixing various tissues are listed in *Manual of Histological Staining Method of the Armed Forces Institute of Pathology*

(3rd edition (1960) Lee G. Luna, HT (ASCP) Editor, The Blakston Division McGraw-Hill Book Company, New York (Exhibit 1)) and in the *Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology* (1994) Ulreka V. Mikel, Editor; Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C. (Exhibit 2)).

In fact, methods of preparing fixed tissue was sufficiently advanced by the time the instant Application was filed that automated tissue fixing machines were commonly patented. Automated tissue fixing technology are claimed in numerous issued US patents including, at least, US 6,058,788 (automated machine for fixing tissues (Exhibit 3)), US 4,834,943 (tissue processor for fixing and embedding resin impregnated specimens (Exhibit 4)), US 4,688,517 (automated tissue fixation and embedding (Exhibit 5)), US 3,889,014 (machine for automatic fixation, dehydration, and clearing of tissue specimens (Exhibit 6)). Other examples of automated tissue processing and fixing machines are reported in 3,526,203 (Exhibit 7); 3,771,490 (Exhibit 8); 3,227,130 (Exhibit 9); 2,959,151 (Exhibit 10); 2,386,079 (Exhibit 11); 2,341,198 (Exhibit 12); 2,157,875 (Exhibit 13); 3,400,726 (Exhibit 14); 2,681,298 (Exhibit 15); and 2,684,925 (Exhibit 16). In summary, methods for preparing fixed treated samples from multiple tissues were well known at the priority date of the instant patent Application and automated machines and methods for fixing tissues have been the subject of numerous patents.

Second, the Examiner asserts that the disclosure provides one working example and fails to disclose any other conditions for performing the claimed method. (Office Action, page 7, first paragraph). Applicants traverse and note that Examples are not required in a Specification. See, MPEP §2164, "[c]ompliance with the enablement requirement of 35 U.S.C. [§] 112, first paragraph does not turn on whether an example is disclosed." The instant specification have provided a working Example and sufficient description throughout the text to practice the claimed invention. Specific teachings that may be found in the Specification is detailed below. For example the process of preparing a sample of fixed treated tissue is described in the Specification on page 16 line 7 to page 17 line 17 and on page 23 line 22 to page 25 line 11. The process of preparing cell lines and tissue for FISH is described in the Specification on page 17, line 19 to page 18 line 22. The process of fluorescence in situ hybridization (FISH) is described

in the Specification on page 18 line 24 to page 21 line 14. In addition, the Example section provides description for at least one method for performing the claimed invention. For these reasons, Applicants assert that the instant specification has provided a working Example and sufficient description throughout the text so that one of skill in the art would know how to practice the claimed invention without undue experimentation.

Third, the Examiner assert that the specification does not disclose the composition of the a solution for deparaffinization and antigen retrieval. (Office Action, page 7). Applicants traverse this basis for rejection because solutions for performing deparaffinization and antigen retrieval was well known and commercially available at the time the instant application was filed (i.e., July 21, 2003). For example, it was known at the time the instant application was filed that "Declere™" solution from Cell Marque or "Reveal™" solution from BioCare Medical can both perform deparaffinization and antigen retrieval steps using just one solution. As support, Applicant submit a material data sheet from Sigma-Aldrich which shows that the Declere™ solution was available since at least August 2001 (See, Exhibit 17, date stamp of August 2001 on the bottom right hand corner). Applicants asserts that it is not necessary for the instant Specification to list the formulation of solutions that are well known and commercially available to those of skill in the art.

Fourth, the Examiner asserted that the specification does not provide "an adequate description of any probe, labeled or otherwise" (Office Action, page 7, first paragraph) as a basis for rejection. It is Applicants' position that the description of probes and labels is immaterial to instant pending claims. The claimed methods and compositions, as embodied in pending claims 1-13 and 33 do not encompass probes or labels and do not contain language directed to probes or labels. It is immaterial whether probes and labels are described in the specification because only the claimed invention is required to be supported by the specification. Since the claimed do not recite probes or labels, the basis of rejection is moot.

For the reasons stated above, it is therefore asserted that the instant application has met the requirements for written description for the claimed invention under 35 U.S.C. 112. Withdrawal of this ground of rejection is respectfully requested.

§ 112 First Paragraph - enablement

Claims 1-13 and 33 stand rejected under 35 U.S.C. § 112 first paragraph as allegedly nonenabling. Applicants traverse.

The Examiner listed two basis for rejecting the claims. Each of the Examiner's basis of rejection is discussed below.

First, the Examiner contends that the claims are not enabled because the claims encompasses using virtually any temperature, pressure and duration and that the disclosure fails to provide conditions under which the claimed method can be practiced for the full genus of cells and tissues. As discussed above, the claimed invention is directed to a method of prepared cell lines and tissues and such methods are well known. Furthermore, methods for pressure cooking a sample are well known. For Example, "pressuring cooking" is described in the review article by Shi et al. (Antigen Retrieval Immunohistochemitry: Past, Present, and Future, J. Histochemistry and Cytochemistry 45:327-43 (1997) (Exhibit 18)) and also by Miller et al. (Heat-induced epitope retrieval with a pressure cooker - suggestions for optimal use. Appl. Immunohistochem. 3:190-93 (1995) (Exhibit 19)) and Norton et al. (Brief, High-Temperature Heat Denaturation (Pressure Cooking): A Simple And Effective Method Of Antigen Retrieval For Routinely Processed Tissues., J. Pathol. 173:371-79 (1994) (Exhibit 20)). In addition, solely in an effort to expedite prosecution, Applicants have amended the independent claims to recite the pressure cooking is performed at a temperature of 125°C and a pressure of between 20 to 24 PSI. For these reasons, Applicants assert that a person of skill in the art would know the proper temperature, pressure and other conditions to practice the invention without undue experimentation.

Second, the Examiner alleged that the specification is not enabled because "it fails to set forth a reproducible procedure whereby the resultant product is used to overcome art-recognized

issues of enablement - namely, a problem set forth in Muhlhahn et al. (US 2004/0038270) associated with cells or tissue detaching from a slide while FISH is conducted" (Office Action, page 9, lines 19-24).

Applicants note that Fluorescence In Situ Hybridization (FISH) is an established technique widely accepted in the art as a valid and verifiable approach for clinical diagnosis and research. Numerous researchers and other persons of skill in the art have successfully used FISH in spite of the alleged problems cited by the Examiner. For Example, numerous patents have been issued for methods involving FISH. See, e.g., US 6,548,259 entitled multiparametric fluorescence *in situ* hybridization (Exhibit 21); US 6,506,563 entitled multiparametric fluorescence *in situ* hybridization (Exhibit 22); US 6,221,607 entitled automated fluorescence in situ hybridization detection of genetic abnormalities (Exhibit 23); US 6,136,540 entitled automated fluorescence in situ hybridization detection of genetic abnormalities (Exhibit 24); US 6,043,037 entitled rapid method for measuring clastogenic fingerprints using fluorescence *in situ* hybridization (Exhibit 25); US 6,007,994 entitled multiparametric fluorescence *in situ* hybridization (Exhibit 26); 5,792,610 entitled method for conducting multiparametric fluorescence *in situ* hybridization (Exhibit 27); and US 5,759,781 entitled multiparametric fluorescence *in situ* hybridization (Exhibit 28). Furthermore, fluorescence *in situ* hybridization (FISH) has been used in a variety of areas of research and clinical diagnostics for over 10 years (See, e.g., Gray, J. W. et al., Curr Opin Biotech 3:623-631 (1992) (Exhibit 29); Xing, Y. et al., In: The Causes and Consequences of Chromosomal Aberrations. I. R. Kirsch Ed. CRC Press, Boca Raton, pages 3-28 (1993) (Exhibit 30)).

Based on the numerous reports of successful applications of FISH, Applicants respectfully asserts that ordinary artisan can practice FISH without undue experimentation in spite of the alleged problems "with cells or tissue detaching from a slide while FISH is conducted" (Office Action, page 9, lines 19-24). In view of the detailed description, working example, and well-known techniques and factors disclosed in the instant application, it is respectfully asserted that the application provides sufficient guidance for the claimed methods

and composition. Withdrawal of the rejection of pending claims 1-13 and 33 is respectfully requested.

§ 103

Claims 1-13 and 33 stand rejected under 35 U.S.C. § 103 as allegedly obvious over Engle and Baschong. Applicants traverse.

There is no motivation to combine Baschong with Engel because the two references are directed to incompatible methods.

Engel is directed to inspection of immunostained tissue section by light microscopy at low magnification of 10x or 40x. See, Engel, page 38, col. 1, first paragraph. Because Engel's method uses light microscopy, it does not use an excitation frequency to stimulate fluorescence and it cannot detect autofluorescence (or any fluorescence). For this reason, autofluorescence cannot be a problem in Engel's method. In contrast to Engel, Baschong is directed to controlling autofluorescence in confocal laser scanned tissue. See, Baschong, title. A person of skill in the art would find no reason to combine the autofluorescence technique of Baschong with Engel because autofluorescence is not a problem and is, in fact, undetectable in Engel's low magnification (10x to 40x) light microscopy method.

There Is No Expectation of Success Even If Engel and Baschong Were Combined

There is no expectation of success even if Baschong is combined with Engle. Baschong have conflicting results and states "the result of the present study highlight the fact that there is no general recipe available for the control of autofluorescence. Success was found in a tactical approach that can be summarized as choice of the appropriate reagent(s) by trial and error . . ." Baschong, page 1571, first column. Since Baschong have made no suggestions as to a method for reducing autofluorescence, a combination of Baschong and Engle, is merely an experiment which, in Baschong's characterization, involves choosing the appropriate reagents by trial and

error. Further, as discussed in the next section, a combination of Baschong and Engel in any event would not lead to Applicants' claimed invention.

Even If Baschong And Engle Were Combined, It Would Not Lead To Applicants' Claimed Methods And Compositions

The claimed invention, is directed to a method used for preparing cell-line and/or tissue samples. In the method, cells are pressured cooked and treated with ammonia-ethanol and sodium borohydride (See, e.g., claims 1 and 33). Other claims are directed to compositions comprising a fixed-treated tissue, ammonia-ethanol and sodium borohydride.

Baschong teaches that a combination of ammonia-ethanol treatment and sodium borohydride treatment is undesirable by stating:

> "[t]o our surprise, treatment with borohydride induced bright autofluorescence in erythrocytes that had otherwise remained inconspicuous (Figure 1F). **This undesirable effect** was slightly diminished by adding ammonia-ethanol (Figure 1G) or sudan [black B] (Figure 1H) and even better if both reagents were combined (Figure 1I). Nevertheless, borohydride treatment once applied, autofluorescence in erythrocytes could not be completely abolished (Figure 1I)." Baschong at page 1567 (emphasis added).

As discussed above, Engel refers to wet heat treatment for paraffin wax embedded tissues and is silent on the suppression of autofluorescence. One of skill in the art, in reading Baschong and Engle, would be discouraged from using a combination of borohydride and ammonia ethanol because Baschong states that the undesirable autofluorescent effect of borohydride treatment "could not be completely abolished."

In fact, the cited references teaches away from the claimed invention. In contrast to the undesirable effects of borohydride and ammonia ethanol, Baschong states "A near to total absence of autofluorescence was attained by treatment with ammonia-ethanol and Sudan [black B]." Baschong at page 1567. Thus, there is no motivation to combine Baschong with Engel to

come up with Applicant's claimed invention. At best, a combination of Baschong and Engel would lead to the use of ammonia-ethanol and Sudan black B which does not contain all the recitations of Applicants' claims.

For the reasons stated above, Applicants believe that the rejection of claims 1-13 and 33 under 35 U.S.C. § 103 was improper and should be withdrawn in view of Applicants arguments.

CONCLUSION

On the basis of the foregoing amendment and remarks, Applicants respectfully submit, that the pending claims are in condition for allowance. If there are any questions regarding this amendment and remark, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

While Applicants believe that no additional fees are due, the Director is authorized to charge all fees that may be due, or to credit any overpayment, to the undersigned's account, Deposit Account No. **50-0311**, Ref. No. **24817-503**, Customer No. **35437**.

Respectfully submitted,

Date: March 30, 2005

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Armed Forces Institute of Pathology

Advanced Laboratory Methods in Histology and Pathology

Ulrika V. Mikel, Editor

Armed Forces Institute of Pathology
American Registry of Pathology
Washington, DC

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Contents

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Immunohistochemistry: Antigen Detection in Tissue

Gary L. Bratthauer and Lila R. Adams

INTRODUCTION

Immunohistochemistry involves the detection of specific chemical substances within tissue by the use of derived antibodies to the substances. Antibodies are applied to tissue sections and allowed to bind to their corresponding antigen. A detection system is then employed to identify the location of these antibodies using marker molecules that can be visually recorded.

Antibodies are produced from the introduction of a specific chemical substance to the immune system of an unrelated species. The immune system has the innate ability to recognize, via specific receptor molecules, virtually any combination of amino acids, carbohydrates, or lipids and respond. This recognition is dependent on many factors, one of which is size. A molecule of several hundred daltons is required to initiate receptor recognition and immune response. This molecule is called the antigen. Many proteins are large enough to elicit an immune response and therefore are antigenic. Many other molecules, or small proteins, are not and must first be attached to a larger molecule. These small molecules can then be recognized by the immune system and are called haptens. When a foreign molecule is introduced to the body, it is recognized as such in conjunction with the particular human leukocyte antigen (HLA) receptors on macrophages. The macrophage digests the molecule and presents certain combinations of external groups of atoms called epitopes on the surface of the cell. The epitope is then brought into contact with helper T-cell lymphocytes, which help present the epitope to B-cell lymphocytes. The B cells synthesize immunoglobulin protein

2

In Situ Hybridization

This chapter covers the in situ hybridization technique on paraffin-embedded material. The procedure can be varied in many different ways. We have chosen to present two versions, each discussed by a different department at the AFIP.

In Situ Hybridization of Viral Inclusions

Robert E. Cunningham

INTRODUCTION

The advances in immunology and molecular biology have revolutionized diagnostic medicine and have greatly contributed to biomedical research as a whole. One of the techniques that utilizes both immunology and molecular biology is in situ hybridization (ISH). In situ is Latin for "in its original place" and hybridization means "to cause the production of a hybrid, a cross"; in this case, a hybrid of single-stranded genomic DNA and single-stranded probe DNA. It accomplishes this by enjoining powerful methodologies from the two systems. The diversity of the immunologic detection capability and the exquisite sensitivity of specific DNA base recognition together provide a new powerful tool for diagnosis and research. Appropriate immunological stains enable recognition of phenotypic changes that may reflect genotypic alterations. ISH can reveal those genotypic changes as well as oncogene expression, chromosomal mapping, infectious disease detection,

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Polymerase Chain Reaction

Ann H. Reid

The polymerase chain reaction (PCR) is used to make large numbers of copies of a specific DNA sequence. Normal human genes are present at the level of two copies per cell, and viral or bacterial pathogens may be present at even lower concentrations. Standard laboratory techniques are unable to detect specific DNA sequences at such low levels. PCR allows the DNA to be copied over and over again (a process called amplification) until it is present in sufficient quantity to be easily detected. PCR can be used to demonstrate the presence or absence of a gene; to detect mutation, amplification, or rearrangement of a gene; and to detect viral or bacterial DNA.

The DNA double helix is made up of two strands, each of which consists of a chain of deoxyribonucleotides. The backbone of the helix is formed by a series of phosphodiester bonds in which the 5' carbon of one sugar is bound to the 3' carbon of the next sugar by a phosphate. Thus, a DNA strand is said to have directionality. In a double helix, one strand runs in the 5' to 3' direction, while its complement runs in the 3' to 5' direction. The nitrogenous bases stack one on top of the other within the helix. There are four nucleotides that make up DNA: two purines, adenine and guanine, and two pyrimidines, cytosine and thymine. Each of these is bound by a hydrogen bond across the double helix with only one partner; thus, adenine is always found paired to thymine, and guanine is always found paired to cytosine. The exclusivity of this binding means that the sequence of one strand of DNA can always be deduced from the sequence of the other. (For more information on DNA structure, see reference 3.) The polymerase chain

4

Preparation of Nuclei for Flow Cytometry

Annette Geissel and Joe L. Griffin

INTRODUCTION

Flow-cytometric analysis, in contrast to slower static methods of DNA quantitation, lets us quickly determine the nuclear DNA content of large numbers of cells. DNA flow studies began in 1969 by Van Dilla et al. Initially these studies were limited to the use of hematopoietic tissues, such as peripheral blood, bone marrow cells, and lymphoid tissues. In the 1970's, methods for extraction of whole nuclei from fresh-frozen and/or fixed solid tissues were developed by various laboratories, which in turn lead to experimentation on fixed, paraffin-embedded tissues. Studies on archival material make use of methods developed in the early 1980's for isolating and staining nuclei from paraffin-embedded tissues (Hedley et al. 1983, Hedley 1989, McLemore et al. 1990, Heiden et al. 1991). Recovery of cells from thick paraffin sections allows retrospective studies on collections of tumors and correlation of flow data with clinical history.

The methods described here reflect our experience and include modifications to previously published methods.

DNA measurements by flow cytometry are based on the ability of certain fluorescent dyes to bind specifically and in direct proportion to DNA under certain staining conditions. The principal dye for staining DNA is propidium iodide, which binds by intercalation (insertion) between the double strands of the DNA molecule. Nuclear fluorescence produced by laser excitation is measured and recorded as nuclei flow rapidly in single file through the laser beam. A computer records individual

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Quantitative Staining Techniques for Image Cytometry

Ulrika V. Mikel

INTRODUCTION

Over the last few decades image cytometry has become a tool increasingly applied to research in pathology. One of its main objectives is to determine the amount of deoxyribonucleic acid (DNA) in tumor cells in order to detect if the cells are aneuploid (have too much or too little DNA) and to determine how rapidly they are dividing or proliferating. The DNA content is determined by the amount of nuclear stain uptake in cell populations. This is done by measuring the optical density of cells stained with absorbance stains, which bind to nuclear chromatin in proportion to the amount of DNA, or the fluorescence intensity of cells stained with fluorochromes, which also bind stoichiometrically, i.e., in a proportional way. As pathologists have become more familiar with the technique, some of them are known to have used image cytometry as an aid in the diagnosis and prognosis of tumors. It is quite possible that the importance of this technique will grow and, in the future, routinely be applied and used in most histology laboratories.

Absorbance cytometry

The most commonly used stain for quantitative evaluation of nuclear DNA is the Feulgen reaction. This reaction is based upon cleavage of the purine-deoxyribose

6

Enzyme Histochemistry of Skeletal Muscle

Carole E. Gregory and Joe L. Griffin

INTRODUCTION

This chapter briefly describes methods for staining frozen unfixed sections of muscle biopsies, as applied in the Muscle Laboratory, Department of Neuropathology, AFIP. Enzyme histochemical stains are usually the most useful diagnostic tools for the pathologist interpreting muscle biopsies. Those stains, with a few nonenzymatic stains used for general morphology and localization, are described in this chapter. While our standard biopsy also includes clamped muscle tissue for electron microscopy and for embedding in paraffin after formalin fixation, those topics are not considered here.

Figures 6–1 to 6–12 illustrate artifacts and stains. At the end of the chapter are references to publications that include many photomicrographs of normal and diseased muscle stained by histoenzymatic and related methods.

Some stains answer a yes-no question about a single enzyme to reveal rare deficiency states. Other stains reveal patterns useful for pathologic interpretation, such as NADH and ATPase to show fiber types (type grouping, type predominance, type-specific atrophy, etc.), esterase for denervation (angular atrophic esterase-positive fibers), ATPase for reinnervation (intermediate-density fibers), and alkaline phosphatase for inflammation.

The differences between type 1 and type 2 fibers are summarized in this memory sentence: "One mighty slow, fat, red ox." Type 1 fibers contain more mitochondria, are slow twitch, contain more fat, predominate in what used to be called red

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Cytopathology Techniques

Tracy L. Raber and Leigh Buckner, III

INTRODUCTION

Over the last several decades, cytology has become a widely accepted and integral component of pathology. It is routinely practiced, even in small laboratories where the cytotechnologist and/or cytopathologist may have to rely on help from the histology laboratory. This chapter is intended as a guide of preparatory techniques in cytopathology for histotechnologists, and other technical personnel, who find themselves helping out in a cytology laboratory. Thus, this is an abbreviated version of methods in cytology and not intended for cytotechnologists.

Cytology deals with the structure, function, multiplication, life history, and pathology of cells. Cytopathology implies changes to cells in disease, and cytology samples are examined for their nuclear and cytoplasmic details. Changes in cell patterns help to separate normal and abnormal cells from each other. Cytology preparations can therefore be diagnostic, or helpful in the diagnosis, of disease.

Cell samples can be collected in several ways. The person who handles cytology specimens must be aware that the preparation of cytology samples varies depending upon the type of specimen and the collection method. Gynecologic material (Pap smears) and fine-needle aspirations are smeared and fixed prior to arrival in the cytology laboratory. They are fixed with alcohol-based spray fixative or 95% ethyl alcohol. Watery specimens, or sparsely cellular specimens, such as urines, cerebrospinal fluids (CSFs), and body cavity fluids must be placed on filters or concentrated for optimal cell recovery. Centrifugation concentrates cells into a pellet from which smears, or a cell block, can be made. Cytocentrifugation

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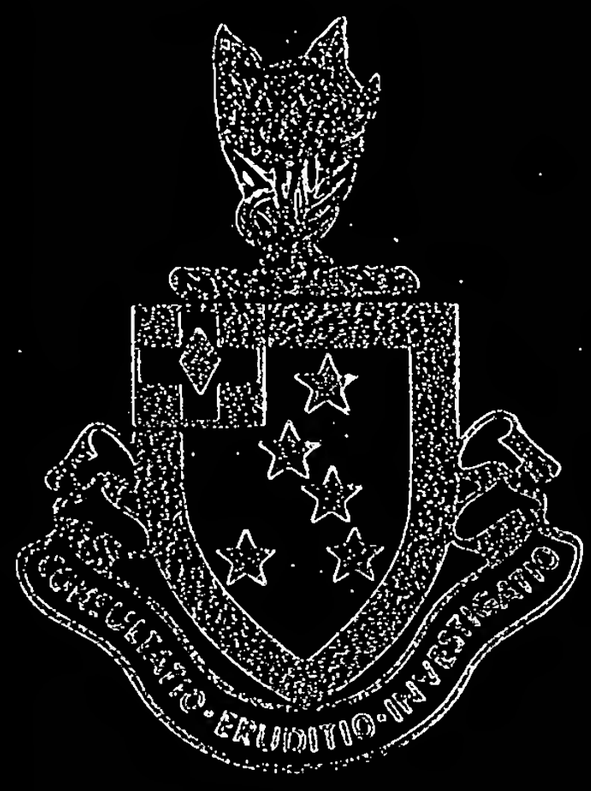
Armed Forces Institute of Pathology



Third Edition

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Lee G. Luna (Editor)

Manual of
HISTOLOGIC STAINING METHODS
of the
Armed Forces Institute of Pathology

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Edited by

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The Blakiston Division

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General References in Histopathologic Technique

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Stain Technology: A Journal for Microtechnic and Histochemistry, The Williams and Wilkins Co., 428 East Preston St., Baltimore, Md. 21202

Contents

Chapter 1

Preparation of Tissue

FIXATION

The foundation of all good histological preparations is adequate and complete fixation. Fixation is required to (1) *prevent* post mortem changes such as putrefaction and autolysis; (2) *preserve* various cell constitutents in as life-like manner as possible; (3) *protect* by hardening the naturally soft tissue, thereby allowing easy manipulation during subsequent processing; (4) *convert* the normal semi-fluid consistency of cells to an irreversible semi-solid consistency; (5) *aid* in the visual differentiation of structure by application of biological dyes and chemicals. To accomplish these objectives the tissue should be placed in the fixative immediately upon removal from the body or as soon after death as possible.

The choice of fixing agent should be determined by the purpose for which the tissue is to be stained or preserved.

Blocks should be cut thin enough so that the fixative will penetrate the tissue within a reasonably short time. To this end the block should not be more than 4mm in thickness and should be immersed in at least ten times its volume of fixative.

Ten percent buffered neutral formalin is the most widely used fixative because it is compatible with most stains. The length of time for fixation depends upon the size of the block and fixative used. It is well to have a clear understanding of the effects of fixation, the time required for complete fixation of specific tissues, and the post fixation handling of tissue specimens.

That many specimens may be ruined, by poor handling subsequent to proper fixation, has been proven. This generally occurs when one fails to realize that different fixatives require varied times to effect complete fixation; and the specimen may require a particular treatment, immediately following fixation, to insure retention of specific staining properties.

Additional useful knowledge, is the action of a simple fixative on different parts of the tissue specimen. A partial list of the characteristics of certain common simple fixatives and their various effects follows, while more detailed information in this regard can be found in: Baker, J. R.: *Principles of Biological Microtechnique,* New York, John Wiley & Sons, Inc., 1958.

CHARACTERISTICS OF CERTAIN AGENTS USED AS FIXATIVES

FORMALDEHYDE, 10% FORMALIN

FORMALDEHYDE, Gas. Formaldehyde is a flammable colorless gas at ordinary temperatures having a pungent suffocating odor. It is very soluble in water (up to 55%) and also is soluble in both alcohol and ether. A very reactive reagent, it combines readily with many substances and polymerizes easily.

FORMALDEHYDE, Solution (Formalin, Formol). A solution of about 37% by weight, of formaldehyde gas in water, usually with 10 – 15% methanol added to prevent polymerization. This solution is the same strength as that known as Formalin 40%.

Chapter 2

Processing of Tissue

DEHYDRATING, CLEARING, IMPREGNATING, AND EMBEDDING

A specimen brought to the laboratory is usually marked with an identifying number or name. Keep this identification with the specimen throughout processing. All identifying marks should be made with a soft lead pencil. Do not use ink or wax pencils.

The surface from which sections are to be cut may be indicated by notching the opposite surface, or by marking it with India ink. An indelible lead pencil may also be used for this purpose. When the tissue is embedded in paraffin, the marked surface of the block is uppermost.

Fixed tissues must be maintained in position by a firm medium so that thin, uniform sections, can be cut. Media suitable for this purpose are paraffin, celloidin, and carbowax.

Processing by the paraffin technic is accomplished most rapidly and gives the best results when thin sections of soft tissue are desired. Since paraffin is not miscible with water, the tissue must be dehydrated and then cleared in solutions miscible with paraffin before impregnation.

Well processed tissue is achieved by a step by step infiltration of the required reagents each preparing the tissue for the one to follow, so that the end result will be a section closely resembling the living state of the specimen. Every cell should be recognizable as to type, enabling the pathologist to focus all his attention on the cell pattern which determines the diagnosis. Properly fixed tissue is essential, as the following steps of processing build upon it.

It is always advisable to remove fixatives before processing. For various methods note remarks under each fixative in Chapter 1.

Dehydration is the removal of all extractable water by a dehydrant diffusing through the tissue, and in the process diluting itself 2 — 4%. Some dehydrants used are tetrahydrofuran, acetone, dioxane, isopropyl alcohol, and ethanol.

Alcohol is the most commonly used dehydrant usually tarting *with 80%*. Exceptions to this are when processing tissue with cavities, cysts, and embryos, which will have *less shrinkage and distortion when started in 60%*. Compact or fibrous tissue, such as muscle, brain, lymph nodes, and glands infiltrate more rapidly and completely in the 60%, *especially when vacuum is employed.* Allow sufficient time in the starting alcohol for complete infiltration of the tissue. The dehydration process continues by upgrading the alcohols to absolute alcohol. Isopropyl alcohol can be used as a substitute for absolute alcohol if necessary, but absolute alcohol is always preferred. *Note.* Isopropyl alcohol should not be used to dissolve dyes and reagents until it has been tested for each use against absolute alcohol.

Acetone provides a rapid method, used sometimes in hospital laboratories, and when required as a "stat" method. The low cost is an asset, but shrinkage and distortion plus a subsequent dryness and hardness which causes cutting problems, are disadvantages to its use.

Chapter 3

Preparation of Sections

CARE AND USE OF MICROTOME KNIVES

The cutting of good sections depends greatly upon practical experience and a complete thorough knowledge of the equipment used. Manual dexterity is a *must*; without it one may face a difficult task in handling the fine manipulative detail required in section cutting. Hurried and inadequate introductory and/or initial training will reflect badly for years afterward; conversely a high standard of training will prevail admirably throughout one's career. *Speed* in performing any phase of histologic technique should *never* be a primary objective since it only leads to unsatisfactory processing, cutting, and staining of the tissue sections. A well trained tissue technician will produce first rate sections in a far shorter time than one who always is aiming primarily at speed.

Since the results produced by histologic technique depend greatly upon the knives used to cut the sections, it is imperative that each technician know how to care for his knife as well as how to use it. A perfect edge on a microtome knife is difficult to describe, however, with a good knife edge, sections of 3 microns in thickness should easily be cut from well-processed, average-sized, tissue blocks. The sections should ribbon off the block in a flat unwrinkled fashion, much as paper comes off a printing press. Microscopically, the section must show no vertical lengthwise scratches or horizontal thick and thin areas. (see Artifacts, page 247). For problems and possible causes in cutting see Fig. 4.

Knife sharpening can be accomplished by mechanical means on one of several kinds of commercial knife sharpeners. However, in the absence of such assistance, it should not be difficult to acquire the skill and ability to keep one's knife in a satisfactory condition using the hand-honing method. The quality of the section produced, more than compensates for the time spent in learning to keep, and in keeping, a knife properly sharpened.

In hand-honing, naturally, good quality stones give the best results. They are expensive, but only the best should be used. The finer the grain in a hone the harder the stone. The yellow Belgian and the Belgian black vein are the finest available anywhere and are, therefore, highly recommended. It is best to purchase a combination hone: Belgian yellow vein and Belgian black vein, mounted back to back.* A liquid medium for sharpening with a hone is necessary, such as household 3 in 1 oil, mineral oil, vegetable oil, or a neutral soap solution. The choice must be left to the technician. At the Armed Forces Institute of Pathology, the neutral soap solution is used because it can be made readily by dissolving household (bar) soap in water.

While honing, the knife should be kept flat, held to the hone by its own weight, with its edge facing the direction of the "heel to toe" motion, under continuous but light pressure (Fig. 5).

*Microtome Knife Hone - Yellow Belgian, Fisher Scientific Co., 7722 Fenton St., Silver Spring, Md. 20910

Chapter 4

Routine Staining Procedures

HEMATOXYLIN AND EOSIN STAINS

Hematoxylin, a natural dye which was first used about 1863, is without doubt the most valuable staining reagent used in histologic work. It has little affinity for tissue when used alone but in combination with aluminum, iron, chromium, copper or tungsten salts it is a powerful nuclear stain. It has polychromatic properties which may be brought out with the proper differentiation. The active coloring agent, hematein, is formed by the oxidation of hematoxylin. This process known as "ripening" takes several days, or weeks, unless it is hastened by the addition of an oxidizing agent such as mercuric oxide, hydrogen peroxide, potassium permanganate, sodium perborate, or sodium iodate. These artificial oxidizers only start the process, so that the solution can be used immediately, but this oxidation process continues slowly over a period of time, during which the hematoxylin retains its staining properties. Once oxidation is complete, the hematoxylin is no longer useful for staining. By increasing the amount of the oxidizing agent the process is much more rapid, thus decreasing the life of the stain so that the amount listed for a given formula should be closely observed. Storage is another factor that has an effect on oxidation. When stored in a dark, tightly sealed container the process is slower, but when in staining dishes exposed to light and air it is markedly increased, therefore, in a staining setup, the solution should be changed at least once a week for consistent staining results.

The most common formulas for staining with hematoxylin are the combinations with aluminum in the form of alum. Those in general use were formulated by Harris, Mayer, Delafield, Ehrlich, Bullard and Bohmer. Sections stained with alum hematoxylins may be counterstained with Eosin, Safranin, Phloxine or other contrasting stains.

The hematoxylins that are combined with iron and tungsten also have their uses. Iron hematoxylin is used in staining myelin and as a nuclear stain in many of the trichrome and other special stain procedures. The one used most extensively in our laboratory was formulated by Weigert. Mallory's phosphotungstic acid hematoxylin (PTAH) is also often used, however not as a nuclear stain.

Although the hematoxylin and eosin stain has been used for nearly a hundred years, a few words of admonition concerning the stain are in order. The word "routine," as applied to the hematoxylin and eosin procedure, should not be used in this connection for Webster defines "routine" as a regular, more or less unvarying procedure. Any one that has worked with the H&E stain knows that many factors contribute to cause some variation in this technic. Examples of this could be: the fixative used, the fixation exposure time, age of staining solution, etc. Therefore, we should not allow the term "routine" to cause the normal amount of laxness usually associated with it.

There are two methods of staining when hematoxylin is employed: Progressive and Regressive.

Chapter 5

Special Techniques

PROCESSING HUMAN TEMPORAL BONES

We are pleased to include in this new edition of histological staining techniques the unaltered procedure for processing human temporal bones. This information was made available to the AFIP by Dr. Stacy R. Guild, Emeritus Associate Professor of Otology of the Johns Hopkins University School of Medicine. A procedure used by Dr. Guild after fifty years experience with human temporal bone histopathology, it is designed to minimize technical artifacts. It represents the distillation of knowledge after experimenting with more than 40 separate histological procedures for human temporal bones. The delicate inner ear membranes require the most careful technical handling to prevent tearing or distortion. The AFIP is grateful to Dr. Guild for making this information available.

INTRODUCTION

The petrous portion of the temporal bone is the housing of the inner and middle ear structures, as well as containing other important tissues such as part of the carotid artery and the facial nerve. Specimens taken for study of the middle ear and inner ear structures should include bone anterior to the internal auditory meatus and posterior into the mastoid region. Between these two landmarks, the desired structures will be found. The specimen block may be removed by making a first cut perpendicular to the tentorial attachment anterior to the internal auditory meatus and a second cut perpendicular to the tentorial attachment 1-1/2 inches postero-lateral to the first. These cuts, if extended 3/4 inch anterolaterally and inferiorly, will contain all of the middle ear and inner ear specimens.

PROCEDURE

1. Place fresh bone specimens in 20% formalin for one hour. Then change to fresh 20% formalin. Tip to allow trapped air to leave the mastoid and other air spaces. Tip slowly, several times, using glass rods or forceps to handle the specimen.

2. Fix in 20% formalin for 24 hours.

Use degassed water to dilute stock formalin. Degassed water is used in order to prevent bubbles from forming on the surfaces of the bone or within the labyrinth. Water may be degassed by subjecting water to a vacuum from a faucet aspirator or a pump for a few hours. Use a thick-walled flask which will withstand the vacuum. For a small number of temporal bones, a 1-liter amount will suffice daily; a 2-gallon jar will be required for large daily usages. After degassing, disconnect slowly from the vacuum; a rapid disconnection will agitate the water with bubble formation. When using the water, transfer by siphon so that it mixes with air as little as possible.

400 ml of fixative is adequate for a pair of bones. In a pint jar add 80 ml of formalin (a 40% solution of formaldehyde in water) to 320 ml of degassed water. Use a tall pint glass fruit jar with glass lid sealed by a rubber ring for each pair of bones. The pair should be kept together.

Chapter **6**

Methods For Connective Tissue

LILLIE'S ALLOCHROME METHOD FOR CONNECTIVE TISSUE

FIXATION. Any well fixed tissue.

TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

SCHIFF REAGENT SOLUTION

(See page 159)

0.5% PERIODIC ACID SOLUTION

Periodic acid	0.5 gm
Distilled water	100.0 ml

0.5% SODIUM METABISULFITE SOLUTION

Sodium metabisulfite	0.5 gm
Distilled water	100.0 ml

WEIGERT'S IRON HEMATOXYLIN SOLUTION

(See page 35)

PICRIC ACID-METHYL BLUE SOLUTION

Picric acid, saturated aqueous	100.0 ml
Methyl blue	40.0 mg

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Periodic acid solution for 10 minutes.
3. Wash in tap water for 5 minutes.
4. Schiff's reagent solution for 10 minutes.
5. Sodium metabisulfite solution for two changes, 2 minutes each.
6. Wash in tap water for 10 minutes.
7. Weigert's iron hematoxylin solution for 2 minutes.
8. Wash in tap water for 10 minutes.
9. Picric acid-methyl blue solution for 6 minutes.
10. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
11. Mount with Permount or Histoclad.

Chapter 7
Methods For Cytoplasmic Granules

DIAZO METHOD FOR ARGENTAFFIN GRANULES

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

1% BRENTAMINE FAST RED B SOLUTION (STOCK)

Brentamine Fast Red B* ..... 1.0 gm
Distilled water ..... 100.0 ml
Keep at 4-5 °C.

LITHIUM CARBONATE SOLUTION (STOCK)

Lithium carbonate ..... 1.36 gm
Distilled water ..... 100.0 ml
Keep at 4-5 °C.

BRENTAMINE FAST RED B SOLUTION (WORKING)

Brentamine Fast Red B (Stock) ..... 5.0 ml
Lithium Carbonate (Stock) ..... 2.0 ml
Mix and let stand for 6-8 minutes at 4-5 °C before use.

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Brentamine working solution at 4-5 °C for 40 to 60 seconds.
3. Rinse in distilled water, two changes.
4. Mayer's hematoxylin solution for 3 minutes.
5. Blue in warm tap water for 5 minutes.
6. Rinse in 95% alcohol, two changes.
7. Dehydrate rapidly in absolute alcohol, then clear in xylene, two changes each.
8. Mount with Permount or Histoclad.

RESULTS

Argentaffin granules	- rust red
Background	- yellow
Nuclei	- blue

*Roboz Surgical Instrument Co., 810-18th Street, N.W., Washington, D. C. 20006

Chapter 8

Methods For Hematologic And Nuclear Elements

WOLBACH'S GIEMSA METHOD

FIXATION. Zenker's or other well-fixed tissues.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

GIEMSA* SOLUTION (STOCK)

Giemsa powder	1.0 gm
Glycerin	66.0 ml
Alcohol, methyl	66.0 ml

Mix glycerin and Giemsa powder. Place in a 60 °C oven for 2 hours. Finally add the 66 ml methyl alcohol.

GIEMSA SOLUTION (WORKING)

Giemsa solution (stock)	1.25 ml
Alcohol, methyl	1.5 ml
Distilled water	50.0 ml

ROSIN ALCOHOL SOLUTION (STOCK)

Rosin, white	10.0 gm
Alcohol, 100%	100.0 ml

ROSIN ALCOHOL SOLUTION (WORKING)

Rosin solution (stock)	5.0 ml
Alcohol, 95%	40.0 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate (see page 41).
3. Wash in running water for 15 minutes.
4. Rinse in distilled water.
5. Working Giemsa solution overnight.
6. Differentiate in working rosin alcohol solution until sections assume a purplish pink color. Check under microscope.

*Stain must be National Aniline Certified Giemsa.

Chapter 9

Methods For Fats And Lipids

SCHULTZ'S METHOD FOR CHOLESTEROL

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

SOLUTIONS

2.5% FERRIC AMMONIUM SULFATE SOLUTION

Ferric ammonium sulfate ..... 2.5 gm
Distilled water ..... 100.0 ml

GLACIAL ACETIC-SULFURIC ACID SOLUTION

Glacial acetic acid ..... 50.0 ml
Sulfuric acid, concentrated ..... 50.0 ml

Add the sulfuric acid slowly, constantly stirring, to the acetic acid in a test tube or flask *cooled with ice. PREPARE FRESH JUST BEFORE USING.*

STAINING PROCEDURE

1. Cut frozen sections and collect in distilled water.
2. Ferric ammonium sulfate solution at room temperature for 3 days.
3. Rinse briefly in distilled water, three changes.
4. Float sections onto the slides and blot dry. Slides may be held at this point until the pathologist is ready for wet reading.
5. Place one drop of *FRESHLY PREPARED* acetic-sulfuric acid solution on the section.
6. Apply coverslip immediately.
7. Examine microscopically within a few minutes.

RESULTS

Cholesterol - green, blue-green, or blue reaction.
Background - colorless

REFERENCE. Schultz, A.: Eine Methode des mikrochemischen Cholesterinnachweises am Gewebsschnitt. *Zbl. Allg. Path.* 35:314, 1924.

OIL RED O IN PROPYLENE GLYCOL METHOD FOR FATS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Frozen sections.

Chapter 10

Methods For Carbohydrates And Mucoproteins

BENNHOLD'S METHOD FOR AMYLOID (CONGO RED)

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns (see Remarks).

SOLUTIONS

1% CONGO RED SOLUTION

Congo red ..... 1.0 gm
Distilled water ..... 100.0 ml

1% SODIUM HYDROXIDE SOLUTION

Sodium hydroxide ..... 1.0 gm
Distilled water ..... 100.0 ml

ALKALINE ALCOHOL SOLUTION

Sodium hydroxide, 1% ..... 1.0 ml
Alcohol, 50% ..... 100.0 ml

MAYER'S HEMATOXYLIN SOLUTION

(See page 33)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Congo red solution for 1 hour.
3. Rinse off excess stain in water, two or three changes.
4. Differentiate in alkaline alcohol solution for 3-5 seconds. Agitate constantly until the background appears clear.
5. Wash in running water for 5 minutes.
6. Counterstain in Mayer's hematoxylin solution for 5 minutes.
7. Wash in running water for 15 minutes.
8. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
9. Mount with Permount or Histoclad.

RESULTS

Amyloid	- pink to red
Nuclei	- blue

Chapter 11

Methods For Pigments And Minerals

HALL'S METHOD FOR BILIRUBIN

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

FOUCHET'S REAGENT

Trichloroacetic acid ..... 25.0 gm
Distilled water ..... 100.0 ml
Mix and add:
10% Ferric chloride ..... 10.0 ml

10% FERRIC CHLORIDE SOLUTION

Ferric chloride ..... 10.0 gm
Distilled water ..... 100.0 ml

VAN GIESON'S SOLUTION

(See page 76)

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Fouchet's reagent for 5 minutes.
3. Wash in running water, then in distilled water.
4. Van Gieson's solution for 5 minutes.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Biliverdin	- green
Collagen	- red
Muscle	- yellow

REMARKS. Bilirubin is oxidized to biliverdin, and stains olive drab green to emerald green, depending upon the concentration of bilirubin.

REFERENCE. Hall, M. J.: *Amer. J. Clin. Path.* 34: 313-316, 1960.

Chapter 12

Methods For Nerve Cells And Fibers

RAMON Y CAJAL'S METHOD FOR ASTROCYTES

FIXATION. Formalin ammonium bromide for 1 day at 37 °C or 10% buffered neutral formalin (see *Note*).

TECHNIQUE. Cut frozen sections at 15 to 30 microns.

SOLUTIONS

FORMALIN AMMONIUM BROMIDE SOLUTION

Formalin, 37 – 40% (Merck's blue label)	15.0 ml
Ammonium bromide	2.0 gm
Double distilled water	85.0 ml

BROWN GOLD CHLORIDE SOLUTION (STOCK)

Brown gold chloride solution (stock)	1.0 gm
Mercuric chloride solution (stock)	100.0 ml

Solution will keep in a dark bottle for many months.

MERCURIC CHLORIDE SOLUTION (STOCK)

Mercuric chloride	0.5 gm
Double distilled water	10.0 ml

Make just before use. Dissolve bichloride of mercury by *gentle* heat.

GOLD SUBLIMATE SOLUTION (WORKING)

Brown gold chloride solution (stock)	10.0 ml
Mercuric chloride solution (stock)	10.0 ml

Mix while mercuric chloride is still warm and add 40 ml double distilled water. A precipitate will form if mercuric chloride solution was not warm enough when mixed. In this case, begin again with the preparation of gold sublimate solution.

5% SODIUM THIOSULFATE (HYPO) SOLUTION

(See page 89)

STAINING PROCEDURE. Use acid clean glassware. Carry frozen sections on glass rod.

1. Rinse in distilled water, two changes.
2. Place sections flat in freshly prepared gold chloride-sublimate solution for 4 to 6 hours in the dark at room temperature.
3. Rinse in distilled water 5 minutes.
4. Sodium thiosulfate solution for 5 minutes.

Chapter 13

Methods For Bacteria, Fungi, And Inclusion Bodies

FITE'S METHOD FOR ACID FAST ORGANISMS

FIXATION. 10% buffered neutral formalin.
TECHNIQUE. Cut paraffin sections at 6 microns.

SOLUTIONS

XYLENE-PEANUT OIL SOLUTION

Peanut oil* ..... 1 part
Xylene ..... 2 parts

ZIEHL-NEELSEN CARBOL FUCHSIN SOLUTION

(See page 220)

1% SULFURIC ACID SOLUTION

(See page 102)

METHYLENE BLUE SOLUTIONS

(See page 218)

STAINING PROCEDURE. Use control slide.

1. Deparaffinize through two changes of xylene-peanut oil solution for 12 minutes each.
2. Drain, wipe off excess oil and blot to opacity. The residual oil helps prevent shrinkage and injury of section.
3. Zeihl-Neelsen carbol fuchsin solution for 30 minutes.
4. Wash in tap water for 3 minutes.
5. Differentiate slides individually with sulfuric acid solution until sections are faint pink, about 1 minute.
6. Wash in running water for 3 minutes.
7. Counterstain lightly with working methylene blue solution.
8. Rinse off excess methylene blue in tap water.
9. Blot and let stand for a few minutes to air dry thoroughly.
10. Dip slides in xylene before mounting.
11. Mount with Permount or Histoclad.

*Matheson, Coleman and Bell, Norwood, Ohio

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The Causes and Consequences of Chromosomal Aberrations

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Chapter

1

Molecular Cytogenetics: Applications of Fluorescence Hybridization to Chromosomal Aberrations and Cancer Genetics

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INTRODUCTION

Advances in cytological hybridization technology have, over the last 20 years, progressed from a laborious and time-consuming approach to detect abundant nucleic acid sequences with low resolution, to an approach which allows quick and highly precise localization of as little as one molecule per cell. Using this technique, it is now possible to detect just a few kilobases of deleted or misplaced DNA anywhere within the genome. These improvements have important implications for human gene mapping as well as for investigations into the occurrence, causes, and consequences of chromosomal aberrations. In addition, while allowing for the determination of linear arrangements of genes on chromosomes, high-resolution *in situ* hybridization also makes possible detailed analysis of their three-dimensional organization and expression within the nucleus. In this chapter we will briefly review the development of this technology and highlight efforts from our laboratory as

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well as others in this area. Various applications to chromosomal aberrations will be considered, with emphasis on cancer cytogenetics. Gene mapping techniques will be presented only briefly since this subject has been recently reviewed (Lawrence, 1990; McNeil et al., 1991; Lichter et al., 1991). Finally, recent developments in applications of fluorescence hybridization to interphase nuclear organization will be considered and discussed in relationship to the generation of specific chromosomal rearrangements.

Technical Development of *In Situ* Hybridization Technology

Early efforts in this field relied on autoradiographic detection of radioactive probes to detect abundant sequences, such as localization of DNA sequences in amplified polytene chromosomes or highly reiterated sequences on metaphase chromosomes (Gall and Pardue, 1969; Evans et al., 1974). Since then, statistical localization of single copy sequences based on autoradiographic grain distributions was described (Harper et al., 1981; Gerhard et al., 1981) and is still widely applied. However, the resolution of autoradiography is limited to large chromosomal segments, and the technique is extremely time consuming, generally requiring weeks for sufficient autoradiographic exposures. Moreover, this approach does not allow localization of a single sequence within a single cell, but requires analysis of grain distributions from 50 to 100 cells. Autoradiography does offer the advantage that cDNA sequences less than 1 kb can be used routinely for statistical localization; however, genomic clones containing repetitive sequences have not been used, presumably because of prohibitively high backgrounds.

Several laboratories have contributed to the development of innovative nonisotopic detection techniques to avoid the shortcomings inherent in autoradiographic methodologies (reviewed in Bauman et al., 1990; Lawrence, et al., 1990; Narayanswami and Hamkalo, 1991; Lichter et al., 1991; McNeil et al., 1991). During the 1980s, several approaches were described, including a method for direct labeling of fluorochromes to RNA probes (Bauman et al., 1981); the incorporation of biotinylated dUTP into DNA probes (Langer et al., 1981; Manning et al., 1975) detected by antibiotin antibodies after hybridization to amplified polytene sequences (Langer-Safer et al., 1982); the use of probes labeled with AAF (*N*-acetoxy-*N*-acetyl-2-aminofluorene) for detection of abundant sequences (Tchen et al., 1984); mercuration of probes (Dale et al., 1975; Hopman et al., 1986); and sulfonation (Verdlov et al., 1974) or direct attachment of enzymes (Renz and Kurz, 1984). Recently a system using digoxigenin-labeled nucleotides detected by antibodies carrying fluorescent or enzymatic tags has become popular (Boehringer Mannheim). Using improvements in the hybridization process (see below), most of these detectors are now known to be capable of detecting single-copy sequences, in some cases as small as 1 kb. Most recently, directly labeled nucleotides have become available (Boehringer Mannheim). Prelim-

inary data indicate that these are clearly effective for detecting centromere sequences (Gerdes and Lawrence, unpublished work); however, single-copy sensitivity has not yet been shown (although it most likely will be in the near future).

Labeled probes have most commonly been detected by fluorescent or enzymatic reporter molecules which recognize a modified probe. The enzymatic detection methods, such as horseradish peroxidase and alkaline phosphatase, require extra steps to produce a visible product; however, they have advantages over fluoresence in that the reaction can be prolonged in order to amplify signals, and the signals do not fade. Fluorescent tags, such as fluorescein or rhodamine, provide the highest resolution possible with the light microscope and can be adapted for multicolor labeling. Probes can also be detected with gold for electron microscopy (see Singer et al., 1989; reviewed in Narayanswami and Hamkalo, 1991).

Nonisotopic methods have been utilized for over a decade. However, due to apparent limitations in sensitivity and reproducibility their use was very restricted, but was successful in detecting highly abundant or amplified sequences (e.g., Wu and Davidson, 1981; Manuelidis et al., 1982). It is only in the last few years that the much greater potential of *in situ* probe technology has been realized, largely due to improvements in the total hybridization process, rather than in the detection systems themselves. Our contributions in this area relied on a rapid quantitative approach (Lawrence and Singer, 1985; Lawrence et al., 1988) devised to allow testing of a vast array of technical parameters, while controlling for the most commonly encountered internal variations. Detection of nucleic acids still within cytological materials is substantially more involved than when they are extracted and hybridized on filters. For analytical purposes, the steps of *in situ* hybridization are best divided into three main components: (1) *preservation* of target sequences in a well-preserved but accessible state, (2) *hybridization* of the probe to the target molecules with high efficiency and without substantial nonspecific adherence, and (3) *detection* of the probe with sufficient efficiency to give a detectable signal, with minimal nonspecific background. Failure of any one parameter in any of these components results in a lower signal-to-noise ratio and a loss of sensitivity. Hence, an analytical approach was adopted to allow simultaneous but separate quantitation of each component (Lawrence and Singer, 1985; Singer et al., 1987; Lawrence et al., 1988).

Our goal for DNA detection was to detect single-copy sequences with hybridization efficiency high enough to provide sister chromatid labeling and, hence, nonstatistical localization within individual cells. While many laboratories had developed different protocols for *in situ* hybridization, it was difficult to know *a priori* which parameters were unnecessary or even destructive, and which were essential for improving results. Using the rapid analytical approach by liquid scintillation counting (Lawrence and Singer,

1985), we were able to identify several key parameters which improved results reproducibly (reviewed in Lawrence, 1990; McNeil et al., 1991). Kinetic studies, for instance, revealed that hybridization of even complex sequences was complete within 3 to 4 h, and that longer hybridization times could cause decreased signal due to RNA degradation. Detection with avidin was found to be dramatically improved by simply changing the buffers used in staining. An extremely important parameter for eliminating the high backgrounds which had plagued the use of nonisotopic probes proved to be probe fragment size after labeling, which has unexpectedly been found to dramatically affect the nonspecific adherence for the probe to cytological material, and the iterative detection of individual molecules. When this hidden variable was controlled, it was then possible to change another key parameter, probe concentration. While the standard procedures called for low probe concentrations, these actually must be extremely high for single sequences ($\geq$5 μg/ml), well above theoretical saturation. Other parameters which significantly influenced the quality of results (often by preserving DNA) are storing slides at -70°C and "hardening" by baking before denaturation; minimizing pretreatments prior to denaturation; omitting RNase A prior to hybridization; testing lots of formamide for neutral pH, correct melting point, and effectiveness for hybridization; monitoring of time, temperature, and pH during denaturation (Lawrence et al., 1988; reviewed in Johnson et al., in press). We found that autoclaving dextran sulfate further inhibits nonspecific sticking of probes. A variety of other steps were tested and found to be unnecessary, such as extensive rinses, proteinase digestion, and acetic anhydride.

Nonstatistical Sequence Detection

Combining the results of these analyses with elements of previous protocols (Harper et al., 1981; Gerhard et al., 1981; Langer et al., 1981), it was shown that it was possible to detect single sequences of a few kilobases by standard fluorescence microscopy using a one-step fluorescein-avidin detection of biotinated probes, without amplification or image-processing procedures (Lawrence et al., 1988). Figure 1A illustrates results, and Figure 2 outlines the protocol used. Perhaps the most critical aspect of this work was that the high hybridization efficiency and low background achieved allowed *nonstatistical* detection of single sequences, in greater than 90% of individual metaphase or interphase cells. The position of a sequence along the chromosome length became immediately obvious in just one metaphase due to the identical labeling of sister chromatids. Because of the extremely high signal-to-noise ratio it was possible to localize *single* sequences within *interphase* nuclei for the first time.

The power of this technology was initially exemplified for analysis of Epstein-Barr virus (EBV) sequences in human lymphoma cells (Lawrence et al., 1988) which revealed the then surprising degree of resolution attainable between closely spaced sequences within decondensed interphase nuclei. Not

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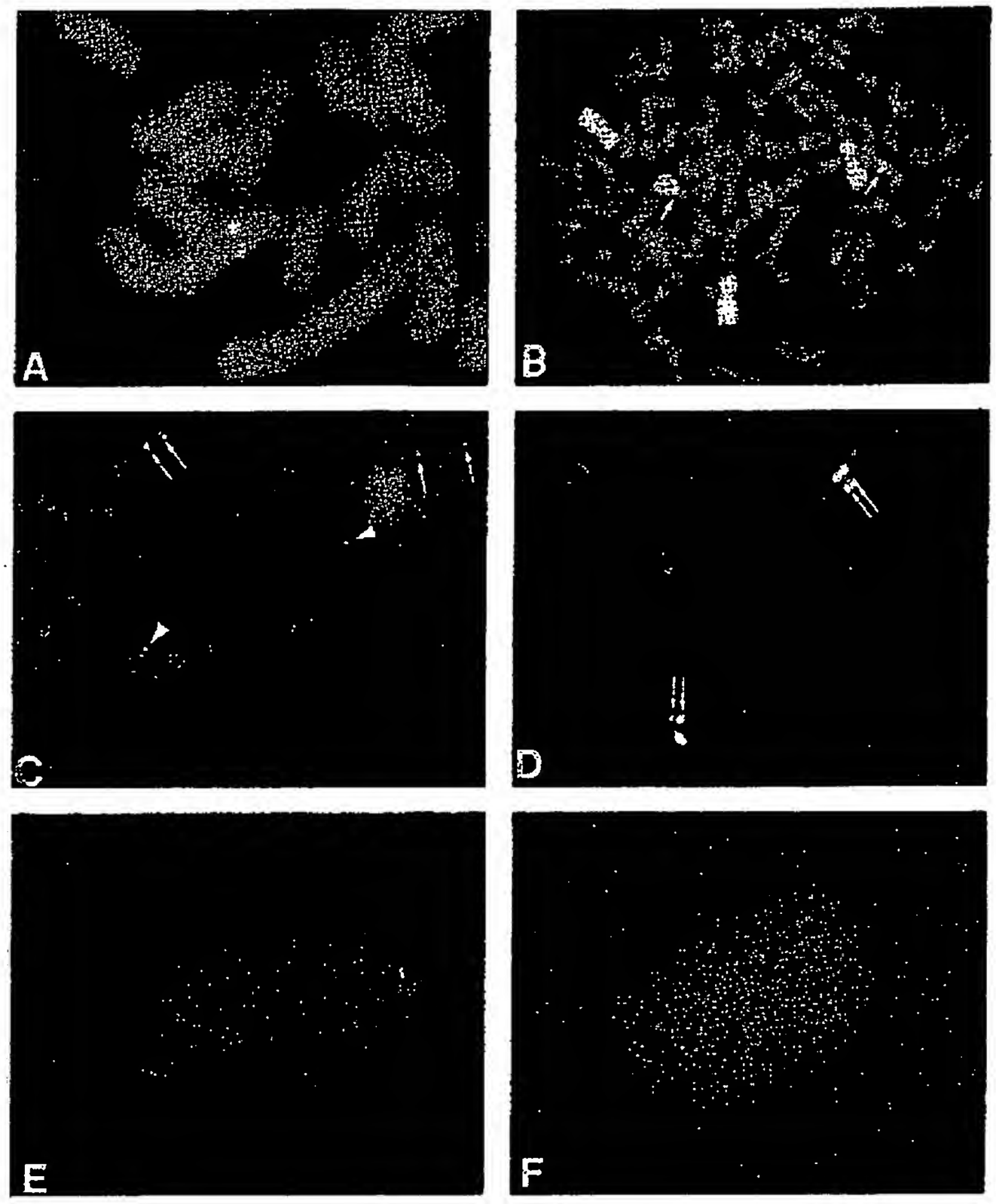

Figure 1. (A) Hybridization to an 18-kb target within the EBV genome, using biotin-labeled probes and a one-step fluorescein-avidin detection. Chromosomes are stained with propidium iodide and visualized using standard fluorescence microscopy. (B) Hybridization of a chromosome-7 library to a tetraploid cell line carrying a translocation involving this chromosome. Two normal chromosomes 7 and two derivative chromosomes (arrows) are observed. (Photograph contributed by K. Wydner and L. Sciorra, Department of Pediatrics, Diagnostic Genetics, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School.) (C) Hybridization of a chromosome-1 sequence to cytogenetic preparations of the Namalwa cell line, illustrating how a cytogenetic abnormality is evidenced by the nonidentical labeling of sister homologues. Note that three signals, rather than two, are observed within the interphase nucleus. The duplicated sequences are approximately 70 Mb apart and are ~5 μm apart at interphase. (D) Simultaneous hybridization to *neu* (erB2) and nerve growth factor receptor cosmid clones which are closely linked and frequently resolvable along the length of less-condensed metaphase chromosomes. On more-condensed chromosomes, they may only be resolvable across the chromosome width. These sequences have been localized to separate bands (17q 11.2-q12 and 17q 21.3-q23), respectively and are ~10 cM apart (~10 Mb). (E and F) Dystrophin sequences separated by ~700 kb are clearly separated and resolvable in ~90% of interphase nuclei. (Figures 1A, C, and D from Lawrence, J. B., in *Genomic Analysis*, Vol. 1, *Genetic and Physical Mapping*, S. Tilghman and K. Davies, Eds., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1–38. Copyright 1990. With permission.)

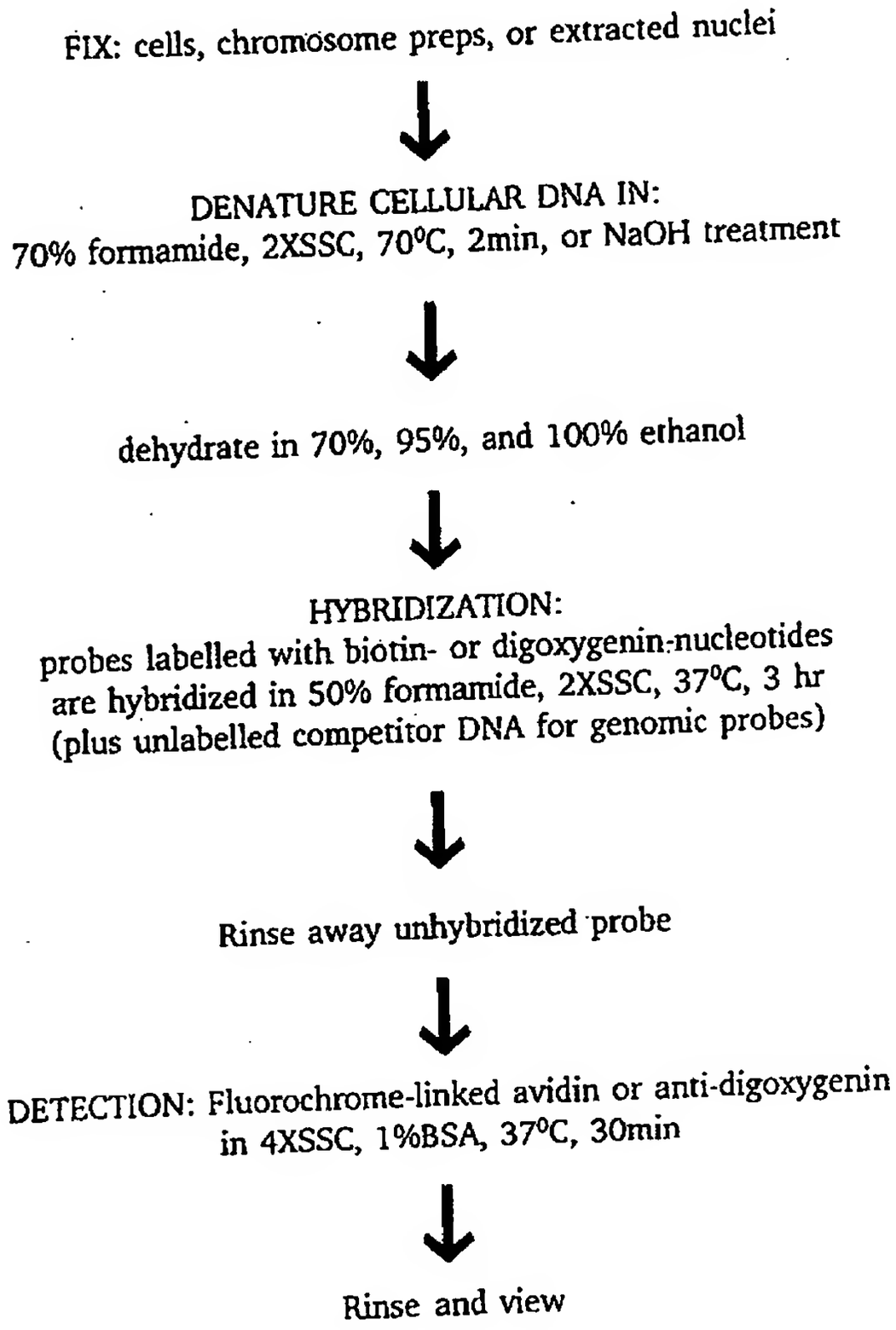

Figure 2. Methodology for hybridization and detection of specific DNA sequences in cytological preparations. (1) Cytogenetic preparations are made by standard techniques using hypotonic solution or methanol-acetic acid. Alternatively, intact cell samples are fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). (2) Denature cellular DNA in 70% formamide, 2 × SSC 70°C for 2 min, or treat samples with 0.2 *M* NaOH, 70% ethanol for 6 min. Then, dehydrate samples in 70, 95, and 100% ethanol briefly. For biotin- or digoxigenin-labeled probes, melt dry probe and unlabeled competitor DNA in 100% formamide for 10 min at 75°C. (3) Hybridize probes to samples at a final concentration of 2.5 to 5 μg/ml (for single-locus probes) in 50% formamide 2× SSC, 1% BSA, and 25% dextran sulfate, 37°C, overnight. (4) Rinse samples in 50% formamide and 2× SSC, 2× SSC, 1× SSC, 30 min each. (5) Detect hybridization with fluorochrome-linked avidin or antidigoxigenin in 4× SSC, 1% BSA, 37°C, 30 min. (6) Rinse in 4× SSC, 4× SSC with 0.05% Triton, 4× SSC, 1×PBS, 15 min each. Further details of these protocols have been reviewed elsewhere (Lawrence, 1990; McNeil et al., 1991; Johnson et al., 1991b).

only were two closely integrated viral genomes detected and resolved, which had escaped previous autoradiographic analysis, but two sequences from either end of a single viral genome, spaced just 130 kg apart, could be resolved as two distinct, closely paired spots for each of the viral genomes (Lawrence et al., 1988). Using two different approaches, it was possible to

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determine the orientation of the two genomes with respect to one another and to estimate the distance between them. First, from the observed interphase order of dim and bright signals and the distance between them, it could be surmised that the viral genomes were in reverse orientation and separated by 200 to 300 kb of cellular DNA. This was done using simultaneous hybridization of two probes of different sizes which produced different intensity signals and corresponded to opposite ends of the EBV genome. A second approach, whereby each of these two probes was hybridized separately and the average distance between them determined, confirmed this interpretation (reviewed in Lawrence, 1990). These results suggested that interphase analysis could be used to determine the order and approximate distance between tightly linked sequences, leading us to propose an approach to gene mapping by fluorescence hybridization termed "interphase chromatin mapping" (Lawrence et al., 1988).

Gene Mapping

An obvious and important application of the ability to detect single-copy genes with fluorescence is to contribute to mapping of the human and other genomes. The resolution of fluorescence, as well as the speed and efficiency of this approach, is far superior to previous autoradiographic techniques which have a limit of resolution on the order of 10^4 or 10^5 kb. While autoradiography has had some advantage in sensitivity over fluorescence, we currently find that fluorescent signals as small as 1 kb can be detected without amplification or image processing, particularly with digoxigenin. Amplification of biotinylated probes (Pinkel et al., 1986) can also be used to detect probes of this size (Fan et al., 1990). However, in much genomic mapping today the need is not to detect smaller probes, but larger ones. Hence, it was very important to show that genomic probes containing repetitive elements could be used. This was initially reported using competition with Cot-1 DNA for detection with peroxidase and interference reflection microscopy (Landegent et al., 1987). Several laboratories then showed that a similar approach worked for fluorescence detection of single-copy phage, cosmid, or Yac clones (Staunton et al., 1989; Trask et al., 1989; Lawrence et al., 1990; Lichter et al., 1990).

For genome mapping, there is a need for alternative physical mapping techniques which provide resolution in the range of 1 to 2 Mb and below, and which allow evaluation of physical distance across a broad range, to help bridge the gap between lower- and higher-resolution techniques. Over the last few years, work from several laboratories (Lawrence et al., 1988, 1990; Trask et al., 1989, 1991; Lichter et al., 1990) has shown that fluorescence hybridization can provide such an approach. Rigorous characterization of the limits and versatility of this technique showed that the ability to resolve and order sequences on chromosomes generally does not extend below 1 to 2 Mb (Lawrence et al., 1990) (see Figure 1D). However, an important finding

was that resolution could be greatly enhanced by analyzing the distance between two sequences in the interphase nucleus, where the chromatin is much less condensed (Lawrence et al., 1988, 1990; Trask et al, 1989, 1991). As described above, this was initially based on the demonstration that one could detect two sequences within an EBV genome separated by 130 kb (Lawrence et al., 1988). Interphase distance was then shown to exhibit a strong correlation with DNA distance over the ranges examined, for both dhfr sequences in Chinese hamster (Trask et al., 1989) and for human dystrophin sequences in normal human cells (Lawrence et al., 1990), providing a needed approach for determining physical order. As illustrated in Figure 1 E and F, sequences within the dystrophin gene separated by 50 kb or more are readily resolved at interphase. Figure 3 schematically illustrates the type of results obtained by analyzing both interphase and metaphase cells, and the legend indicates the distances over which each approach is most successful for gene ordering.

Another major effort from several groups has involved the adaption of fluorescence hybridization with chromosome banding techniques (Figure 4), some of which allow probes and chromosomes to be identified simultaneously (Viegas-Pequignot et al., 1989; Lawrence et al., 1990; Fan et al., 1990; reviewed in McNeil et al., 1991). The details of various banding procedures as well as the strengths and limitations of both metaphase and interphase mapping have been extensively reviewed recently (Lawrence, 1990; Lichter et al., 1991; McNeil et al., 1991). Included in these is a discussion of dual or multicolor labeling techniques, and Figure 2 outlines our protocol for two-color detection, as detailed previously (Johnson et al., 1991a).

APPLICATIONS FOR ANALYSIS OF CYTOGENETIC ABERRATIONS

It is only recently that methodology for applying nonisotopic cytological hybridization for the analysis of cytogenetic aberrations has begun to realize its full potential. The speed, convenience, and precision of nonisotopic probe-labeling techniques make this technology applicable for diagnostic as well as research purposes. With the increased quality and reproducibility of results that can now be achieved, a new area of "molecular cytogenetics" has emerged, whereby standard karyotypic analysis is directly coupled with molecular biology. Until recently, the field of cytogenetics has been limited to the analysis of relatively gross chromosomal aberrations based on banding patterns. Standard karyotypic analysis, while allowing a survey of the complete chromosome complement, can only discern deviations in whole chromosomes or chromosome segments containing approximately 10 Mb or more of DNA. It is now possible to detect specific genetic defects 1000- to 10,000-fold smaller cytologically, and eventually it may become possible to detect

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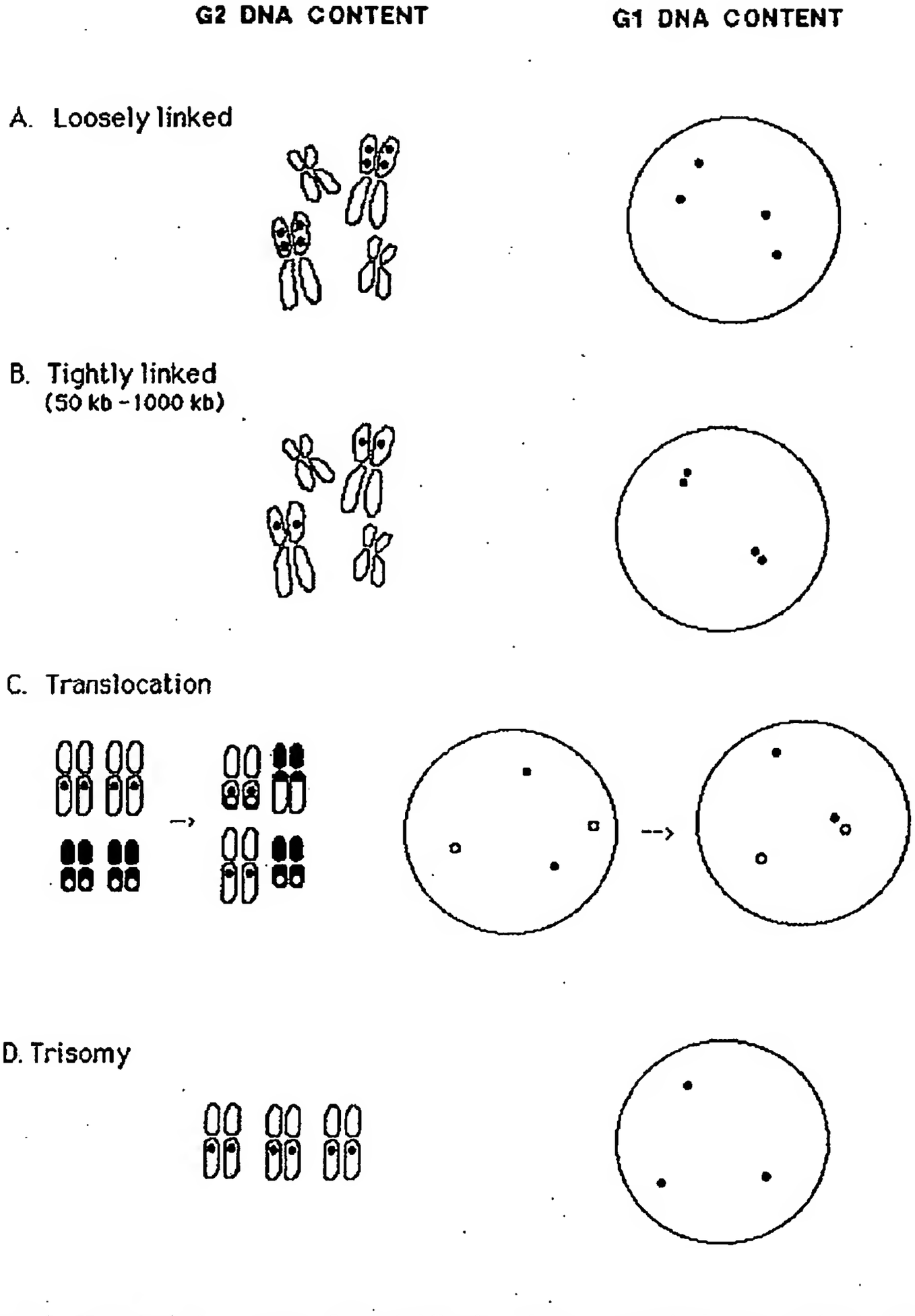
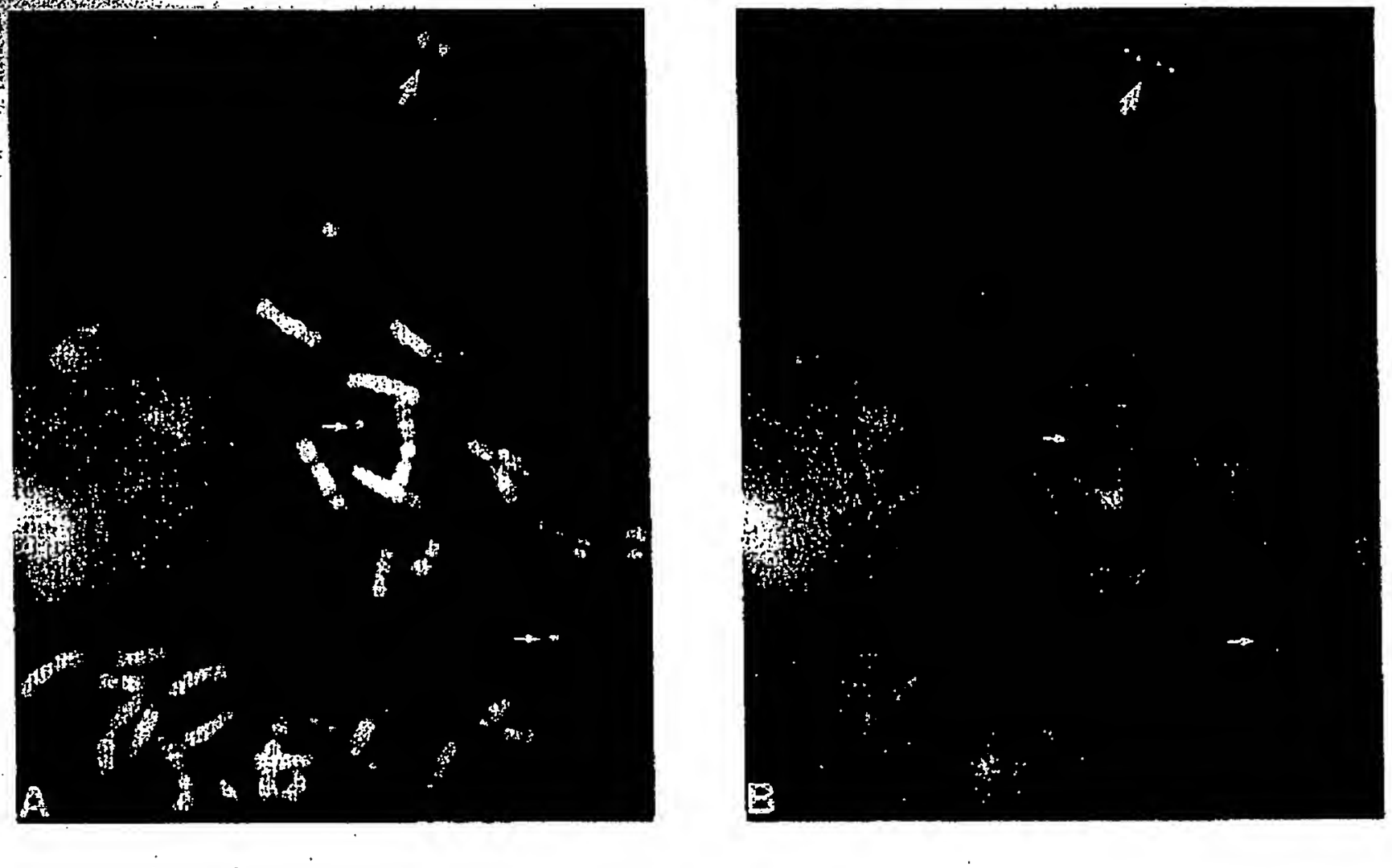


Figure 3. Hypothetical outcomes after hybridization of one or two single-copy probes to interphase and metaphase cells. (A) Loosely linked sequences will be resolvable along the chromosomes length and show only a distant pairing within interphase nuclei. (B) When sequences from the same chromosomal region become closer, they will no longer be resolvable at metaphase but will still be clearly visualized as closely paired signals within decondensed interphase nuclei. (C) Hybridization patterns for normal (left) and translocated (right) metaphase and interphase cells. Solid and open circles represent signals from two pairs of different chromosomes. In normal cells the interphase nucleus shows a widely dispersed variable distribution of four hybridization signals. In contrast, in cells carrying the translocation, the fusion of two different chromosomes consistently gives a pair of close solid and open signals in both interphase and metaphase nuclei. (D) In the case of trisomy, three chromosomes showing sister chromatid labeling will be seen in metaphase, and three hybridization signals will be visualized at interphase.



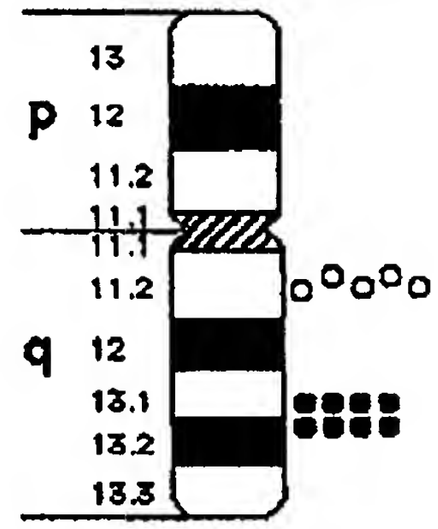


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Figure 4. Localization of the PTP1B gene and p107 gene by fluorescence detection of *in situ* hybridization. (A) Diaminophenylindole staining of metaphase chromosomes from normal human lymphocytes. The large arrow points to the homologous pair of chromosome 20s. The small arrows indicate chromosome 19, distinguishable based on the staining pattern. (B) Fluorescence-avidin detection of hybridization with the biotin-labeled PTP1B genomic DNA probe. Both homologues of chromosome 20 label on both sister chromatids. (A and B from Brown-Schimer, S. et al., *Proc. Natl. Acad. Sci. U.S.A.* 87, 5148, 1990. With permission.) (C) Schematic representation of chromosome 20 with a summary of the PTP1B gene and p107 gene placement based on analysis of banded metaphase chromosomes and simultaneous localization of PTP1B gene and p107 gene on chromosome 20. (From Ewen et al., *Cell* 66:1155, 1991. With permission.)

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even smaller defects. In the hunt for specific disease genes, a molecular cytogenetic defect in the gene itself might be revealed, because any aberration resulting in nonidentical labeling of homologous chromosomes is readily apparent by this technique. Clearly, the technology has value for clinical research into the etiology of genetic disease, but the potential for clinical diagnostics is also strong. A few areas of potential application to cytogenetic abnormalities are summarized below.

Hybridization of chromosome libraries, chromosome-specific centromere probes, or even small single-copy sequences can all make readily apparent monosomies or trisomies of specific chromsomes (see, for example, Figure 1C). The most common clinical abnormalities in this category are trisomies 21, 13, and 18 and the sex chromosome abnormalities XO, XXX, XX, and XYY. With the use of a collection of several probes for the above chromosomes with multiple labels, it should be possible to screen for several aneuploidies at once. Although all of these aberrations are readily detectable by standard karyotypic analysis, an advantage of the *in situ* hybridization approach is that it can be done directly in interphase cells, making it unnecessary to culture cells, such as amniotic fluid cells or peripheral blood lymphocytes. Cremer et al. (1986) described the use of "interphase cytogenetics" for detecting trisomy 18, and other investigators have used this approach with chromosome libraries, for example, for demonstration of trisomy 21 (Lichter et al. 1988; Pinkel et al. 1988). Although initially done with chromosome libraries, the more discrete signals generated by smaller probes such as cosmids or cosmid sets are more easily enumerated (Lawrence, unpublished observations).

Chromosome libraries or specific repeats can also be used for the detection and characterization of translocations, as shown in Figure 1B. This strategy is applicable to the detection of specific translocations known to characterize particular types of cancers. In addition, translocations that appear to be balanced can be analyzed much more precisely using a battery of probes for a specific chromosomal region. Translocations may be even more readily detected at interphase using two probes known to flank the breakpoint, which will be detected unusually close together (on the same chromosome) when the translocation is present.

Submicroscopic deletions involving a few kilobases or more of DNA are readily apparent by *in situ* hybridization, which allows for many potential applications. Carrier detection is one example, since heterozygous gene deletions are difficult to identify by Southern blot analysis because heterozygosity is manifest only as the change in intensity of a band, often difficult to discern. With *in situ* hybridization, on the other hand, heterozygosity is readily apparent as the total absence of signal on one homologue. The presence of signal on the other homologue serves as an internal positive control. Because hybridization efficiency is high (>90%), deletions can be confidently identified by analysis of just a few cells. Duchenne's muscular dystrophy is an excellent example of how *in situ* deletion detection might be

useful. In this disease, the most common molecular defect has been identified as deletions of 6 kb or more in the dystrophin gene (Koenig et al., 1987). Once a specific deletion is identified in an affected male by filter hybridization with different probes, female family members can easily be screened for the same deletion using metaphase or interphase analysis (Lawrence et al., 1991). Affected individuals themselves might be screened with a small battery of probes for the most common deletions. As specific defects in inherited diseases are identified and characterized and the sensitivity and convenience of these techniques increases still further, the number of potential applications will grow. As considered below, one major area in which this may prove to be very important is in the diagnosis and study of cancer, which is essentially a genetic disease of somatic cells.

APPLICATIONS OF FLUORESCENCE HYBRIDIZATION TO CANCER GENETICS

Genetic changes are now well recognized to have a fundamental involvement in the complex process of cancer development for many, if not all, cancers. The specific genetic changes which contribute to cancer involve altered gene expression as a consequence of gene loss resulting from translocations or deletions, or smaller mutations such as point mutations. The ability of fluorescence *in situ* hybridization to provide more precise molecular cytogenetic analysis promises to make a significant contribution to the understanding and detection of genetic changes in cancer. Here we consider some of the ways in which *in situ* hybridization can be useful in investigating the etiology and diagnosis of cancer, after briefly reviewing some of the major types of genetic changes known to be involved.

The first consistent chromosome change in human cancer was the Philadelphia (or Ph^1) chromosome in chronic myelogenous leukemia (Nowell and Hungerford, 1960), which occurs as the result of a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] (Rowley, 1973). There are now at least 70 recurring translocations that have been detected in human malignant cells (reviewed in Trent et al., 1989). For example, chromosome translocation breakpoints have been described in the mixed parotid gland tumors at 3q21 and 3q25; (Mark et al., 1980). A consistent deletion is another genetic factor frequently observed in human cancer, with deletion of a specific region correlated with a particular tumor tissue type. These rearrangements may be multiple and complex. For example, in human colorectal carcinomas, 5q, 17p, and 18q are all found to be altered in many tumors (Baker et al., 1989), and a putative tumor suppressor gene, Mcc, has been found at 5q21 (Kinzler et al., 1991).

In the progression of tumor formation, it has been found that either the activation of oncogenes or the inactivation of tumor suppressor genes, or

both, play an important role. Proto-oncogenes, or the counterparts of viral oncogenes, have been found to be related to some chromosomal rearrangements recurrent in certain types of human cancers (Bishop, 1987). Mapping of these proto-oncogenes to human chromosomes revealed that many are located at the breakpoints implicated in different cancers (Heim and Mitelman, 1987). For example, the MYC proto-oncogene is found located at band 8q24, which is the breakpoint in Burkitt's lymphoma (Dalla-Favera et al., 1982), and the RAF1 oncogene has been mapped to band 3q25, which is often broken in mixed parotid gland tumors (Bonner et al., 1984). Hence, chromosomal changes may directly or indirectly induce the activation of oncogenes.

The tumor-suppressor genes are a group of genes which have important functions in the regulation of normal cell proliferation and development and whose presence in normal cells is required to prevent the emergence of a tumor. Loss of function of tumor suppressor genes has been found or is suspected in many different cancer types. A predominant example is the development of retinoblastoma, now known to involve loss of both allelic Rb genes mapped on the long arm of chromosome 13q14. Deletions of chromosome 13q are a consistent finding in patients with constitutional chromosome abnormalities who had a high incidence of retinoblastoma (Francke, 1976; Friend et al., 1986; Lee et al., 1988; Harbour et al., 1988; Horowitz et al., 1990). Individuals with the hereditary form of retinoblastoma have been found to have inherited the loss of one copy of the gene in the germ line, and then a somatic event inactivates the normal allele inherited from the other parent. It is now clear that Rb inactivation plays a key role in the pathogenesis of several commonly occurring tumors, including small cell carcinoma of the lung, bladder cancers, and breast carcinomas (T'ang et al., 1988; reviewed in Marshall, 1991).

One of several recent developments which have opened new opportunities for understanding the role of genetic factors in cancer is the expansion of capabilities for precise gene mapping. There are a number of reasons why the assigning of genes to chromosomes has received considerable attention. A key route to finding genetic factors involved in cancer is to identify genes affected as a consequence of specific chromosomal rearrangement that occur consistently in certain tumor types. Therefore, gene mapping will provide the essential information regarding the chromosomal location of genes involved in cancer and in the precise identification of the gene itself, which can be approached by several strategies.

Mapping Tumor Suppressor-Gene Candidates to Chromosomes

One approach in which *in situ* hybridization has potential to contribute to cancer genetics is to identify the chromosomal locus of a known gene which, based on its biochemical function, may be a putative tumor suppressor

or oncogene. It can then be determined if that locus correlates with known specific chromosomal changes implicated in tumors. Two examples in which our laboratory has applied this strategy, as part of collaborative efforts, involve genes encoding tyrosine phosphatases (Brown-Shimer et al., 1990) and p107, a protein with biochemical similarities to Rb (Dyson et al., 1989; Ewen et al., 1989; DeCaprio et al., 1989). This has resulted in the identification of two candidate genes possibly involved in myelogenous leukemia (Brown-Shimer et al., 1990; Ewen et al., 1991). This possibility must then be further investigated by analysis of whether those genes are specifically mutated in this cancer.

Biochemical studies have shown that increases in tyrosine phosphorylation by receptor tyrosine kinases lead to altered cell growth; hence, tyrosine kinases are recognized as important in the biochemistry of cancer. Similarly, loss or reduction in activity of a phosphotyrosyl phosphatase will also result in net increases in the phosphotyrosine content of cells and potentially lead to oncogenic transformation. *PTPG*, the gene for protein-tyrosine phosphatase γ has been mapped to chromosome region 3q21 and is found frequently deleted in renal cell carcinoma and lung carcinoma (LaForgia et al., 1991). Thus, the chromosomal location of tyrosine phosphatases has been of increasing interest (Brown-Shimer et al., 1990; LaForgia et al., 1991).

To determine if PTPase 1B mapped to any chromosomal site known to correlate with a specific cancer, a genomic clone was isolated and used as a probe for fluorescence hybridization to banded metaphase chromosomes. As illustrated in Figure 4, it was found that the human PTP1B gene is a single-copy gene located on chromosome 20q13.1–q13.2. This was of interest because the long arm of chromosome 20 was known to show deletions in or near this region in a significant fraction of cases with myeloid disorder (Davis et al., 1984), raising the possibility of involvement with myelogenous leukemia. Another putative tumor suppressor gene p107 was found to map to 20q (Ewen et al., 1991), possibly closer to the most consistent deletion in myelodysplastic disease (Yunis et al., 1988). The p107 protein has been of much interest as a potential tumor suppressor because it was found to share functional properties with the tumor suppressor product, RB, and binds to SV40 large T antigen and adenovirus E1A (DeCaprio et al., 1989; Dyson et al., 1989; Ewen et al., 1989). A cDNA clone for human p107 has been obtained, and its coding sequences map to chromosome 20q11.2. Using two-color hybridization it was directly shown that p107 is proximal to the centromere relative to PTPase 1B (Ewen, et al., 1991). Hence, these two genes provide good markers as well as candidate genes to better characterize the deletions of 20q in the tumors, which is often difficult with just banding procedures.

After mapping tumor suppressor genes to chromosomes from normal human samples, the next step is to identify if there is an allele loss in cancer cells. With clones of the possible tumor suppressor genes, *in situ* hybridization can be used to determine the number of copies of the gene in the

cancer cells compared with normal cells. In some cancers, the inactivation of both copies of the gene may be caused by different types of chromosomal changes. For example, in familial retinoblastoma, only a small fraction of the patients show germ-line loss of the first copy of the gene at the cytogenetic level. In most cases, no obvious cytogenetic abnormality can be detected and the inherited mutation is either a small deletion or even just a point mutation. However, the somatic event that causes the loss of the second copy is usually a large deletion, recombination, or total chromosome loss. For such changes, *in situ* hybridization will pick up the second, large deletion of the gene, but may not be able to detect the smaller abnormality in the first gene copy. In contrast, both copies of the gene in normal cells will be detected. This is essentially the same type of analysis for "heterozygosity" testing as described above, demonstrated for carriers of constitutional deletions in the dystrophin gene.

Search for Unknown Genes at a Known Chromosomal Breakpoint

Another strategy for which high-resolution *in situ* hybridization is useful is in searching for an unknown gene at the precise site of a known chromosomal breakpoint. This generally involves screening libraries of cosmids or yeast artificial chromosomes (YACs) from whole chromosomes or chromosome segments to find those clones closest to the breakpoint. Double-label fluorescence hybridization can be extremely valuable in searching for sequences near a deletion or breakpoint. The search generally would begin by identifying the two closest flanking markers for a specific deletion from previously mapped genes or RFLPs. Commonly, these flanking markers will be on the order of 5 to 10 Mb apart, an enormous distance when looking for a single gene. The two flanking markers can both be labeled in either red or green, and then probes for unknown sequences from that chromosome differentially labeled in the opposing color. The position of the clone of interest, as being between or outside of the two flanking markers can then be evaluated by *in situ* hybridization of the three probes simultaneously to metaphase and interphase cells. We have found this approach useful in searching for sequences near a translocation breakpoint flanked by markers 6 centimorgans (~6 Mb) apart (D. Shapiro and J. Lawrence, work in progress).

In searching for sequences at the breakpoint of chromosome 11q23 translocated in human acute leukemia, a series of YAC clones containing known genes on 11q23 were used as probes to hybridize to chromosomes from patients with acute leukemia (Rowley et al., 1990). What has been found is an intense labeling on the normal chromosome 11 and a less intense signal on the rearranged chromosomes as well as on the translocation partner. Hence, the YAC appeared to be split because of the translocation, indicating that the critical sequences at the breakpoint were within the YAC. This

greatly facilitates the identification of the gene, by limiting the area of the "walk" to identify coding sequences.

Molecular Cytogenetic Detection of Deletions, Translocations, or Other Chromosomal Rearrangements Associated with Specific Tumors

In the inherited forms of retinoblastoma, the absence of genetic material inherited from one of the parents can be detected by the loss of heterozygosity for markers flanking the locus. Since similar events uncovering recessive somatic mutations occur in the sporadic forms of retinoblastoma (Cavenee et al., 1983), the consistent loss of heterozygosity at specific loci in cancers may indicate deletion of tumor suppressor genes. As described above, heterozygous gene deletions may be difficult to identify by Southern blot analysis; however, with *in situ* hybridization submicroscopic deletions involving 1 to 2 kilobases or more of DNA are apparent as the total absence of signal on one homologue. The presence of signal on the other homologue serves as an internal positive control. Alternatively, fluorescence hybridization can be used to detect the amplification of specific sequences. This is evidenced as an increased number of signals for what is normally a single-locus probe, or, in some cases, increase intensity of specific signals. For example, it has been shown that *neu* oncogene is amplified in breast cancer (Salmon et al., 1989). Hence, the same technique that detects deletions should also be useful to identify gene amplification, which may be important for tumor prognosis.

To detect the specific translocation known to characterize particular types of cancers, chromosome libraries (see Figure 1B), specific repeats, or a group of probes for a specific chromosomal region can be used. Translocations may also be detected on metaphase chromosomes by comparing the location of probes which flank the site of the breakpoint on a given chromosome, as schematically represented in Figure 3C. For analysis of more easily obtained interphase cells, translocations may also be detected using two probes, one from each of the chromosomes involved in the translocation, which will be detected unusually close together (on the same chromosome) when the translocation is present (Figure 3C). In the case of bcr-abl fusion in chronic myelogenous leukemia, the fusion involves a translocation t(9;22)(q34;q11) to produce the characteristic Philadelphia (Ph^1) chromosome. The bcr probe and abl probe were labeled with two different fluorescence colors (red and green), and *in situ* hybridization was used for detection of the fusion in metaphase and interphase cells (Tkachuk et al., 1990). In contrast to the normal metaphase with abl and bcr at chromosomes 9 and 22, respectively, the bcr-abl fusion is shown by a very close two-color pair located in the Ph^1 chromosome. In interphase nuclei, one red and one green signal from chromosomes 9 and 22, respectively, were located randomly in normal interphase cells but in cells carrying the translocation appeared as a

red-green doublet, with the separation between the two <1 μm, indicating the presence of the bcr-abl fusion gene.

Analysis of Viral Integration Sites

Nonisotopic *in situ* hybridization also makes possible the visualization and analysis of individual viral genomes within single cells. The introduction of viral genetic material into human chromosomes is implicated in the etiology of certain diseases, including different type of cancers. For example, HPV (human papilloma virus) is associated with several malignancies including nasopharyngeal carcinoma and Burkitt's and other B-cell lymphomas arising in immunosuppressed patients. While it was previously widely believed that integration of EBV occurred only in a few aberrant cell lines and was not important in the transformation process, using *in situ* hybridization as well as Southern blot analysis indicated that when EBV infects activated or proliferating B-cells it persists as a single integrated copy (Hurley et al., 1991). This finding then raises the interesting issue of to what extent integration could be playing a role in cancer formation. This finding, as well as the detection of episomal vs. integrated genomes, is illustrated in Figure 5. The fluorescence *in situ* hybridization can readily demonstrate single-copy integrated genomes in metaphase cells due to the identical labeling of the sister chromatids in the same chromosome. In contrast, cells containing episomes, which are always present in multiple copies, should demonstrate multiple single hybridization signals, apparently randomly associated with the chromosomes (Hurley et al., 1991). This enhanced ability to investigate the localization of viral genomes with respect to cellular genes is likely to be important for understanding the potential role of viral integration in specific chromosomal rearrangements or cancer.

Nuclear Organization

The new genre of more powerful nonisotopic *in situ* hybridization technology makes possible not only a more refined analysis of genes on chromosomes, but also opens the door for investigations into the higher-level organization of the interphase nucleus. Efforts to completely describe and understand complex genomes will ultimately require investigations into their three-dimensional organization in their functional state within the nucleus. While this has primarily a cell biological or developmental significance, it may also prove relevant to understanding the causes of some specific chromosomal aberrations. For instance, translocations between different chromosomes are not observed as random, but there is a propensity for certain chromosomes to be found recombined with one another, which could possibly reflect, in part, higher-level chromosome organization.

While much is understood about the nucleosome structure of chromatin (reviewed in Georgiev et al., 1981; and in Weisbrod, 1982), little is known

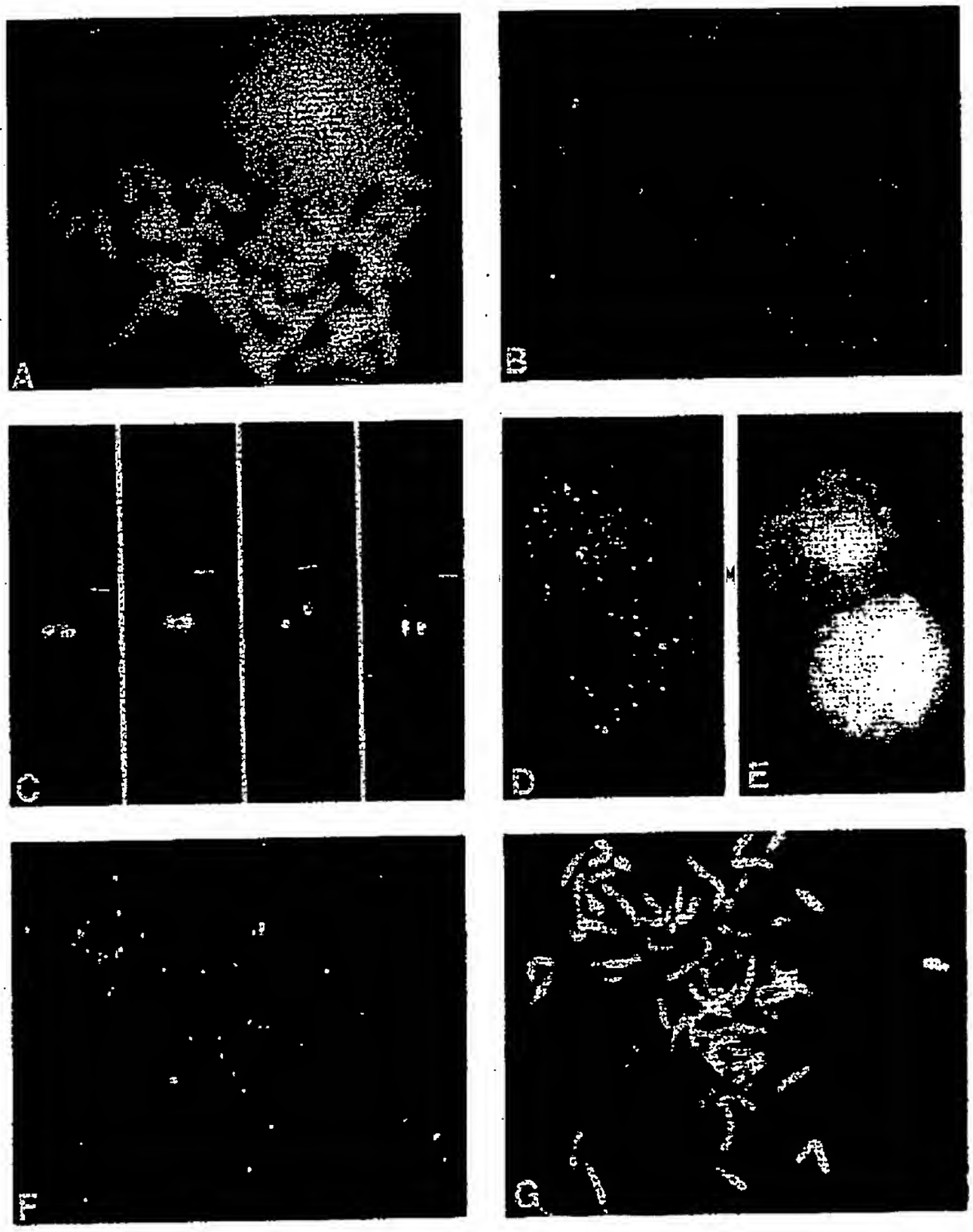

Figure 5. Detection of integrated and episomal viral genomes (A–C) Fluorescein hybridization signals using a *Bam*H1 W probe to the EBV genome, showing sister chromatid labeling of integrated EBV genomes. C shows that the same integration site is observed in identical positions in different cells of a chromosomal cell line. (D–G) The detection of episomal EBV genomes. (D) DNA hybridization within interphase nuclei of a cell line shown by Southern analysis to carry numerous episomal genomes. (E) DAPI DNA staining of nuclei in D. (F) Metaphase figures show numerous signals, but not in obvious sister chromatid labeling and in highly variable patterns. (G) DAPI of metaphase in F. (From Hurley, E. A. et al., *J. Virol.* 65, 1245, 1991. With permission.)

as yet about the higher-level organization of chromosomes and genes within the nucleus (Manuelidis and Borden, 1988; Hamkalo and Rattner, 1980). A three-dimensional order of chromatin within the interphase nucleus has been suggested by a variety of cytological observations (Comings, 1968, 1980; Manuelidis, 1985; Cremer et al., 1982; Hochstrasser and Sedat, 1987; Chung et al., 1990), some dating back over 100 years (Rabl, 1885). However, our understanding of nuclear organization has remained at a preliminary stage,

restricted primarily to investigations of satellite sequences (Manuelidis, 1984) or nuclear landmarks, such as the nucleolus. Such studies have clearly reinforced the cell type-specific higher-level organization of the genome; however, more refined analysis can now be done using a methodological tool which makes possible localization of specific single-copy genes as well as their cognate RNAs. Initial application of this tool revealed that the EBV genome is confined to an inner sphere of the nucleus representing only 50% of the volume, strongly indicating a sequence-specific, higher-level order within interphase chromatin (Lawrence et al., 1988). Even in randomly rotated suspension cells, the propensity for some sequences to be peripheral while others are confined to the nuclear interior has been readily observed for a host of other genes. For example, the *neu* oncogene has an interior localization in peripheral blood lymphocytes, whereas the dystrophin gene localizes at the extreme nuclear periphery, even in male cells where there is no Barr body (Lawrence et al., 1990). While there is clearly a nonrandom orientation to genes within nuclei, there appears to be substantial plasticity with regard to their precise localization (Lawrence, Coleman, and Xing, unpublished observations). This is also suggested by work demonstrating that the relative position of the two homologous chromosome 1 centromeres is highly variable (Van Dekken et al., 1990). The localization of expressed EBV sequences to the nuclear interior in Namalwa lymphoma cells is of particular interest in light of earlier suggestions that active chromatin localizes around the nuclear periphery (Hutchinson and Weintraub, 1985; Blobel, 1985; Puck et al., 1990).

Recent results provide strong evidence for the compartmentalization of the nucleoplasm into functional domains. For several years it has been reported that immunofluorescence for snRNP antigens of the RNA processing type produces clustered patterns within interphase nuclei (Fakan et al., 1986; Ringertz et al., 1986; Spector, 1990) although it had not been fully accepted whether this pattern is bona fide. Our laboratory has recently found that total poly(A) RNA, constituing 90% of mRNA, is localized within 10 to 20 discrete interphase domains (Carter et al., 1991). It was further shown that these "transcript domains" are enriched in snRNP proteins, making it highly likely that these are sites of mRNA processing. Other results indicate that these domains are nonrandomly positioned with respect to the underlying genome, raising the possibility that they are sites of mRNA transcription (Carter et al., 1991). A major goal of work in this area in the next several years will be to determine if active genes are clustered and, if so, if functionally related sequences or sequences from particular chromosomes are consistently localized together in specific cell types. The feasibility of a functional association of genes on different chromosomes is clearly established by the nucleolus, which represents the functional interaction of sequences on ten different chromosomes.

Work from several labs indicates that an individual chromosome does not extend throughout the entire nucleoplasm, but concentrates in a more

restricted space (Cremer et al., 1986; Pinkel et al., 1988; Lichter et al., 1988). It remains to be determined whether specific chromosomes are consistently positioned with respect to one another, which evidence indicates would occur in a cell type-specific manner. If so, this could in part explain why specific chromosomal pairs are most commonly translocated to one another in specific cell types. Another likely explanation for this is that the activation of oncogenes or deactivation of tumor suppressor genes which occurs as a consequence of a particular chromosomal rearrangement confers a selective advantage only in specific cell types. In this scenario, the frequency of different translocations is the same in all cells, but different ones are selected for in different cell types. It is perhaps likely that both of these mechanisms play a role in producing the higher cell type-specific distribution of chromosomal aberrations.

Another important feature of high-resolution *in situ* hybridization technology which has already provided insights into nuclear structure and has potential to contribute to investigations of genetic disease, is the ability to detect primary nuclear transcripts from expressed genes. Fluorescence visualization of specific viral RNAs has revealed very localized, often curvilinear "tracks" of nuclear RNAs up to 5 μ long (Lawrence et al., 1989, Figure 11B). This strongly suggests a highly structured nuclear interior in which RNA is not freely diffusing, and recent work indicates that this RNA may be tightly associated with a nonchromatin fibrillar matrix (Xing and Lawrence, 1991). Other potential applications for nuclear RNA detection may involve investigations into genetic disease; aberrant gene expression can result from a defect at one of several steps from gene to active protein. In the case of Duchenne's muscular dystrophy, a significant fraction of genetic defects in this gene (other than the ~60% known to be large deletions; Koenig et al., 1987) may result from defects in the enormous degree of processing that the dystrophin transcripts must undergo. Nonisotopic *in situ* hybridization provides a potential tool for detecting any chromosomal or genetic aberration that results in defective RNA processing.

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